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SYNCHRONOUS GROWTH OF *CLOSTRIDIUM BOTULINUM* TYPE F, STRAIN LANGELAND

A THESIS

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SYNCHRONOUS GROWTH OF *CLOSTRIDIUM BOTULINUM*

TYPE F, STRAIN LANGELAND

Approved:

Chairman

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SUMMARY

Clostridium botulinum is an anaerobic spore-forming bacillus which produces the most potent type of toxin known to man. Toxins produced by the six known types of Clostridium botulinum cause a neuro-paralytic disease called botulism, which is usually contracted by the ingestion of certain foods contaminated with the growing organism. The ability of this organism to form spores and to produce toxin is of major concern to public health agencies and to the food industry.

A limited amount of research has been reported in the literature on sporulation and the formation of toxin molecules by Clostridium botulinum. This investigation sought methods of synchronizing the growth of vegetative cells of Clostridium botulinum, type F, strain Langeland in the hope that the events which lead to spore and toxin formation might be delineated. Such information would contribute to basic scientific knowledge and possibly provide some insight into the means of eliminating botulism as a threat to public health.

Synchronous growth of vegetative cells was accomplished by germination synchrony of a spore inoculum in TSB^{+} at 30 C. The degree of synchrony was estimated by calculating a synchronization index SI = 0.55 for the first synchronous division and SI = 0.30 for the second step. Transfer into fresh TSB^{+} after the first doubling in the number of vegetative cells following outgrowth of the spore inoculum resulted in an improved synchrony of SI = 0.69 and SI = 0.52 for the first and second divisions, respectively. A third step was only indicated. No synchronous

growth was obtained by using the stationary phase method which consisted of repeated transfers of cultures from specific times in the stationary phase of growth into fresh TSB^+.

Synchronized growth was achieved as a result of cold shocking exponentially growing, asynchronous cultures at 4 C for 45 minutes followed by reinoculation into fresh TSB^+ at 30 C with an estimated SI = 0.48 for the first division and SI = 0.21 for the second. No synchronized growth was attained by cold shocking stationary phase cultures.

From the information gained in the investigation it is concluded that the degree of synchronous growth following germination synchrony may be sufficient to: (1) aid in the study of the initiation of DNA transcription and translation at the time when dormant spores of Clostridium botulinum, type F, strain Langeland make the transition to vegetative cells, and (2) permit the delineation of events which lead to the formation of the toxin molecule. The study of synchronized growth, as a result of cold shocking, is expected to show: (1) the effect(s) a physical agent may have on the physiology of spore and toxin formation, and (2) the interrelationships of DNA and cell division, and RNA and protein synthesis.

CHAPTER I

INTRODUCTION

This thesis is a discussion of attempts to synchronize the growth of Clostridium botulinum, type F, strain Langeland by (i) germination synchrony, (ii) the stationary phase method, and (iii) cold shocking cultures taken from the stationary and the exponential phases of growth. The reasons this study was performed were as follows:

Clostridium botulinum is a Gram-positive, anaerobic bacillus which forms endospores and is catalase negative. These characteristics place the organism into the following taxonomic scheme still accepted by most bacteriologists*:

Division I:	Protophyta
Class II:	Schizomycetes
Order IV:	Eubacteriales
Family XIII:	Bacillaceae

The organism produces the most potent type of toxin encountered by man. This toxin is responsible for a neuro-paralytic disease called botulism usually contracted by ingesting certain foods contaminated with the growing organisms. This disease affects both man and animals but the incidence of botulism food poisoning is relatively rare, a fact which tends to conceal the potential danger of the organism to the general public. Once contracted, the disease often results in death by circulatory failure and respiratory paralysis.

*Bergey's Manual of Determinative Bacteriology, 7th Edition.

Historical Background

The history of botulism dates back to the ninth century. At that time, Emperor Leo VI of Byzantium forbade the eating of blood sausage because of its harmfulness to health (Dolman, 1964). The causative agent of botulism was isolated in 1896, when van Ermengem of Ghent, Belgium, recovered an anaerobic, spore-bearing bacillus from a piece of ham which had been implicated in the death of three persons; the organism was also isolated from the spleen of one of the victims. Van Ermengem found that the culture filtrates, after injection into laboratory animals, produced characteristic and often fatal paralyses. He compared the symptomatology of the victims of this particular incident to the sausage-poisoning syndrome described by physicians in southern Germany in the 18th century. Van Ermengem proposed that the name Bacillus botulinus, derived from the Latin word botulus meaning "sausage", be given to the newly isolated organism (Van Ermengem, 1897).

During the following decades several types of botulogenic organisms were isolated and described, all essentially alike in their biochemical characteristics and in producing typical paralytic symptoms in man, animals, and fowl but distinctly different in their immunological reactions. In 1904, a wax-bean salad caused the death of eleven among twelve consumers in Darmstadt, Germany. The causative organism, isolated by Landmann (1904), resembled van Ermengem's bacillus. Leuchs (1910) succeeded in producing antisera in horses against these two strains and showed that their respective toxins were immunologically distinct. Although these two prototype cultures were soon lost, Burke (1919) confirmed the existence of two different types of botulism which she designated type A and

and type B. It is generally believed (Meyer and Gunnison, 1929) that van Ermengem's strain was a non-proteolytic type B and the Darmstadt strain a type A.

During the 1920's Bengston (1922) in Washington, D. C. and Seddon (1922) in Australia discovered two sub-types of C which they designated type C_a and type C_b, respectively. In South Africa, Theiler, et al. (1927) isolated type D from animal carcasses. Both types C and D have caused heavy losses in many parts of the world among aquatic wild birds ("western duck sickness," type C_a), cattle ("Midland cattle disease," type C_b; "lamziekte," type D), and horses ("forage poisoning," type C_b).

The discovery of type E botulism was described in the literature by Gunnison, et al., (1936). Their prototype E strains had been obtained from Kushnir in Russia who failed to describe them until 1937. Type E is so far known to affect primarily man and is most commonly transmitted by food products of fish and marine mammals.

A sixth immunologic type of Clostridium botulinum was isolated by Møller and Scheibel (1960) from a sample of homemade liverpaste which had been implicated in the death of one person on the Danish island of Langeland. The organism was similar to Clostridium botulinum type A in saccharolytic and proteolytic properties, but its toxin was not neutralized by antitoxins to types A, B, C, or D. The culture was more extensively studied by Dolman and Murakami (1961)and designated type F on the basis of its distinctive immunologic reaction although these investigators discovered that large amounts of type E antitoxin were capable of cross-neutralizing the type F toxin. The incidence on the island of Langeland is the only recorded fatal outbreak of type F botulism to date. A second incidence was reported in

California, 1966, when three persons contracted type F botulism after eating home-made venison jerky. All three survived. Thus, at the present time, serotypes A, B, C, D, E, and F are described in the literature of which type F has been the most recent addition.

The late identification of type F has been attributed by Dolman and Murakami (1961) to its low frequency in nature, although recent publications indicate that the organism is not restricted to the locality of its original occurrences and that the incidence within a particular area may be quite high. In 1965, a second isolation was made from samples of marine sediments which had been collected off the coasts of Oregon and California (Eklund and Poysky, 1965). A year later, a third culture was isolated from the gills and viscera of a sockeye salmon caught in the Columbia River (Craig and Pilcher, 1966). In contrast to the non-proteolytic character of the latter two isolates, Williams-Walls (1968) succeeded in isolating a proteolytic strain from a blue crab (Callinectes sapidus) taken from the York River near Chesapeake Bay, Virginia.

The type F toxin has also been reported to be present in mud samples from a small stream in eastern North Dakota (Wentz, et al., 1967) as well as in a mixture of viscera and gills from a fish caught in the Atchafalaya River, Louisiana, (Ward, et al., 1967) as determined by toxin neutralization patterns. The causative organism itself was not isolated by these investigators, however.

Botulism: The Disease And Its Treatment

The toxin of Clostridium botulinum is known to act specifically on the peripheral nervous system by blocking the transmission of impulses from nerve to muscle. There is evidence that the toxin interferes with the

output of acetylcholine from the ends of cholinergic nerves at the neuromuscular junction either by preventing the release of acetylcholine or by combining with it after its release (Van Heyningen and Arseculeratne, 1964; Brooks, 1964; Zacks, et al., 1962). Although slight symptomatic differences exist, dilated fixed eyes, dry mucous membranes, abdominal distention, constipation, difficulty in swallowing and speech, and progressive respiratory paralysis are symptoms common to all types of human botulism.

The incidence of death may vary greatly depending to a large extent on the immunologic type of the organism. Death or survival also depend on the condition of the food at the time of consumption, the amount eaten, and the speed with which the symptoms are diagnosed and anti-toxin therapy is initiated. Specialized foods prepared by certain ethnic groups, for instance, are especially conducive to the growth and toxin production of Clostridium botulinum, type E. Thus, there is "izushi", a fermented raw fish salad considered a delicacy in Japan. Alaskan Eskimos prefer "muktuk", consisting of beluga flippers either dried or preserved in seal oil. Among the Indians of the northwest Pacific Coast "salmon egg cheese", or "stink eggs" are a favorite food, and in Norway salted trout, or "rakefish", is the most frequently implicated food dish. The mortality rate for type E botulism is between 30 and 40 percent; that for type B strains, most often associated with meat and meat products in Europe, is about 20 percent. In the United States, where home-canned vegetables and fruits have been the most common vehicle and type A the most frequently involved toxin, the case fatality rate is about 70 percent.

Protection against botulism poisoning or treatment of it, once it

has been contracted and recognized at an early stage, is afforded either by type-specific or by polyvalent botulinus antitoxic sera. Toxoid for active immunization against types A, B, C, D, and E, however, is available only in a pentavalent form to qualified, high risk laboratory personnel and only in small amounts at a very few locations in the United States; no toxoid has yet been developed for type F (U. S. Department of Health, Education and Welfare). Furthermore, therapy of the disease is hampered by the general unfamiliarity of physicians with the clinical manifestations of botulism, and the unavailability of simple laboratory tests to make a differential diagnosis.

Relation to Food Processing

Few cases of botulism were reported in the United States between 1950 and 1962. Then, in 1963, outbreaks of type E occurred in Tennessee, Alabama, and Michigan. Nearly one half of the cases, in contrast to previous outbreaks, were related to commercially processed and distributed food including tuna, liver paste, and smoked fish. Sudden outbreaks such as those in 1963 point not only to the potential danger of Clostridium botulinum to human life but also to the devastating economic effect the disease may have on certain kinds of food industries due to the loss of public confidence in their products. At present, prevention of growth of Clostridium botulinum and toxin production in commercial foodstuffs is accomplished primarily by thermal processing (Perkins, 1964).

The vegetative forms of Clostridium botulinum and the toxins they produce are heat labile and therefore readily destroyed by commercial heat processes. The spore forms, however, are quite resistant to heat as well

as radiation treatment, although considerable differences in these characteristics exist among the different types. In calculating "safe" margins for thermal processing of canned foods, the canning industry utilizes the results of extensive studies by Esty and Meyer (1922) on the heat resistance of more than one hundred strains of type A and B spores. Esty and Meyer found that thermal destruction of 6×10^{10} spores of the three most resistant strains of type A and B known and tested by them required 4 minutes at 248 F and 330 minutes at 212 F (in phosphate buffer at pH 7), and that the death rate of the spores between these two temperatures was logarithmic. Extrapolating their data to 250 F, which has been adopted by the canning industry as the conventional reference temperature, the minimum reference lethality time became 2.78 minutes. Greater lethality times were recommended for products with high fat or carbohydrate contents.

The actual processing time for a canned product is the sum of the minimum lethality and the so-called "lag time", which is the time it takes the slowest heating point in a can to reach retort temperature. This lag time, of course, is dependent on the size of the can and the type food it contains. Although it is unlikely that sixty billion of the most heat resistant spores of Clostridium botulinum will ever be found in a single can, this conservative approach to thermal processing is continued by the canning industry. This criterion recognizes the facts that commercial processing conditions cannot be controlled as well as laboratory experiments and that occasional mechanical or human errors may occur under production conditions.

For more than ten years, irradiation processing has been under

intensive investigation by the Army and the Atomic Energy Commission as a possible tool for (i) preserving food products and (ii) making them safe for public consumption. The objectives have been, in order of increasing radiation dosage: sprout inhibition, insect disinfestation, pasteurization, and sterilization. Unfortunately, the dose needed to destroy Clostridium botulinum spores in order to achieve commercial sterility has an adverse effect on the taste and texture of many foods. In addition, any preformed botulinal toxin is not deactivated and certain pathogenic viruses, such as the Lansing strain of the polio virus, will survive the sterilization dose necessary for Clostridium botulinum spores. Nevertheless, irradiation processing, perhaps in combination with chemical treatment, might have large scale industrial application in preserving foods which do not lend themselves to heat sterilization or pasteurization such as bacon, luncheon meats, smoked ham and fish, fresh marine products, fruits and vegetables, and the new experimental high protein concentrates such as dried fish meal.

Both the Army and the Atomic Energy Commission claim that low doses or ionizing radiation greatly increase the shelf life of food products, prevent spoilage, and destroy insects and microorganisms without loss of nutrition and flavor. However, the claim that irradiated food is also safe for human consumption is disputed by the Food and Drug Administration (FDA). This government agency is charged, among other things, with the responsibility of approving the effectiveness and safety of new food additives, and use of ionizing radiation is considered a part of the Food Additives Amendment of the 1968 Federal Food, Drug, and Cosmetic Act. The FDA contends that present data do not demonstrate the safety of most irradiated foods

to the satisfaction of the law (agency). The data do not answer satisfactorily the questions about (i) emission of radioactivity, if any, from irradiated foods, (ii) the effect of irradiation on vitamins, and (iii) the possible formation of radiomimetic compounds which are known to be carcinogenic and mutagenic. As a result, only potatoes and wheat flour have been approved for irradiation processing with the requirement that these products be clearly labelled as radiation processed (Code of Federal Regulations, Title 21). To date, these foods have been produced for the military but not for the public market (Jamison, 1968).

The effectiveness of thermal, radiation, or chemical food preservation processes is dependent upon the characteristics of each type organism. There is extensive information available on Clostridium botulinum types A and B, and an increasing amount for type E; little is known about type F. Continuous research is being supported by the canning industry itself, and by various government agencies, out of an awareness of the shortcomings of present preservation processes and inadequacies in our knowledge of the characteristics of all the types of botulinal organisms. Public health officials, for instance, are greatly concerned about some of the consequences which the practice of irradiation pasteurization or sterilization may have on the radioresistance of microorganisms. Thatcher (1964) reported that the progeny of certain irradiated cultures of E. coli could tolerate 15 times the lethal dose of the parent cultures and were capable of transmitting that tolerance even after two years. Similarly, when spores of Clostridium botulinum were subjected to a radiation dose that destroyed 90 percent of the population and the survivors were again exposed to radiation, one could select for a radioresistance which was twice

that of the original spores. This resistance was carried over from the spores to the vegetative cells and again to newly formed spores. These findings strongly indicate that multiple irradiation permits the evolution of greater radioresistance in microorganisms. In the case of Clostridium botulinum, it is also quite conceivable that the trait of greater resistance to radiation energy may occur by natural mutation since the organism is known to have a high mutation rate (Dolman and Murakami, 1961).

Fruitful Research Areas

Considering the description so far given of botulism, two characteristics of the causative organism seem to be most important for continued investigation in laboratory experiments. These characteristics are spore formation and toxin production. The objective of laboratory research should be not only to acquire knowledge about the organism for the sake of knowledge but also to gather a body of information from which practical applications could be derived for the food industry so that botulism may be eliminated as a potential threat to public health.

In developing the idea for the thesis presented here, it seemed that systematic studies on spore and toxin formation might best be approached by growing synchronous cultures of Clostridium botulinum. In most cases, information found in the literature on the biosynthesis of cellular components and products has been obtained using exponentially growing cultures. Since these consist of a mixture of cells at various stages in the division cycle, the information yields only average values for each single cell. In a synchronous culture, however, the events occurring in a single cell are sufficiently amplified to permit analysis with conventional biochemical tools. A synchronous culture thus provides an experimental system

which not only allows the investigator to measure the synthesis of macromolecules qualitatively and quantitatively but also enables him to determine precisely at which time the synthesis occurs.

Studies on the kinetics of enzyme formation in synchronous cultures, for instance, have revealed valuable information about the relationship between gene position and the time of enzyme synthesis, the accessibility of the genome to transcription and the possible mechanisms of transcription and translation. In his discussion of the present knowledge on enzyme synthesis in synchronous cultures, Mitchison (1969) attempted to classify the pattern of synthesis into two broad groups, each subdivided again into two categories. The first group consisted of enzymes which were synthesized only at a particular time in the division cycle of a cell, the time being characteristic for each enzyme. Synthesis was then discontinued. Most enzymes that have been identified and examined so far fall into this group, strongly indicating an ordered sequence of transcription. The first category of this group was represented by "step enzymes". These were stable following synthesis and thus formed patterns which resembled "steps". The second category comprised "peak enzymes" which soon lose their activity because of inactivation or molecular instability. Enzymes of the second major group were formed continuously, either in an exponential or linear fashion. In the latter case, a constant rate of synthesis was maintained until a characteristic point in the cell cycle was reached, at which time the rate was doubled.

Spore Formation

In synchronous cultures, spore formation is an ideal system for studying the correlation between enzyme and protein synthesis and the

appearance of new structures. Sporulation involves a series of sequential, integrated biochemical events and reactions under genetic control. Morphological and internal structural changes can be followed by microscopic examination of stained preparations, thin sectioning, and electron microscopy. Available evidence suggests that actually two processes occur during sporulation: de novo synthesis of spore proteins and entrapping of vegetative cell protein by the invagination of the initial spore membrane (Murrell, 1967; Spudich and Kornberg, 1968).

Aerobic Sporeformers. Studies on the extensive modifications in the biosynthetic capability of the bacterial cell preceding and accompanying sporulation have primarily been done on B. subtilis (Freese et al., 1969; Takahashi, 1969; Doe et al., 1969; Spudich and Kornberg, 1968; Fortnagel and Freese, 1968; Hanson et al., 1964), B. megaterium (Tono and Kornberg, 1967; Imanaka et al., 1967; Martin and Foster, 1958; Storck and Wachsman, 1957; Grelet, 1957), B. cereus (Baillie and Norris, 1962; Gollakota and Halvorson, 1961; Nakata and Halvorson, 1960; Young and Fitz-James, 1959; Grelet, 1957), and B. licheniformis (Thorn and Stull, 1966; Leitzmann and Bernlohr, 1965), because of the susceptibility of these organism to transformation and transduction. Current evidence on the genetics of sporulation suggests that the sporulation process is controlled by a relatively large number of unlinked genes randomly distributed along the chromosome (Takahashi, 1969, 1965a, 1965b). In contrast, there is also evidence that the first stage in sporulation (Murrell, 1967) may be regulated by a cluster of closely linked genes (Hoch and Spizizen, 1969; Spizizen, 1965).

Considering that sporulation is such a complex biochemical event requiring the synthesis of many new enzymes and proteins not normally found

in the vegetative cells, the suggestion has been made that the spore genome may be quite large containing at least 100 structural and functional genes (Halvorson, 1965). The exact order in which the biochemical and cytological changes take place suggests a sequential reading of the genome. However, information is absent on the first gene which is read to initiate sporulation. Neither is it known how further reading is regulated. Certain physiological changes, such as depletion of nutrients, the reduction of growth or protein synthesis, the production of a sporulation factor(s) or an antibiotic, have been implicated in triggering the sporulation process (Murrell, 1967).

Although there has been modest success in synchronizing the growth and sporulation of aerobic sporeformers by various methods, only the approximate times at which particular macromolecules are produced have been established. Improvement of growth and sporulation synchrony are therefore still a prerequisite for those investigations which attempt to find the exact order and regulation of the physiological and biochemical changes associated with the formation of a bacterial spore.

Anaerobic Sporeformers. Attempts to investigate the physiology and biochemistry of sporulation of the genus Clostridium have been made by Leifson (1931), Kaplan and Williams (1941), Wynne (1948), Collier (1957), Halvorson (1957), Collier and Murty (1957), Lund, et al., (1957), Perkins and Tsuji (1962), Day and Costilow (1964a, 1964b), and Bergere and Hermier (1965a, 1965b). To my knowledge, only Collier, Halvorson, and Day and Costilow attempted to synchronize growth of their cultures. Collier (1957) and Halvorson (1957) succeeded in establishing "rapid and simultaneous sporulation" of Clostridium roseum as evidenced by the sudden rise in spore count

and dipicolinic acid synthesis at the onset of the stationary phase of growth in the final culture. Day and Costilow (1964a) claimed reasonable synchrony of Clostridium botulinum 62-A by observing a "high percentage of the cells in each of the following morphological stages by microscopic examination: (i) vegetative, (ii) swollen cells, (iii) forespores (swollen cells containing a refractive body with diffuse edge), and (iv) highly refractile spores."

Although the investigations into the sporulation process of Clostridia have been small in number and restricted to basic physiological and metabolic activities, the results are in reasonable agreement with those reported for aerobic sporeformers. Information on sequential biochemical events which may lead to the mapping of their genetic loci (as has been done for a number of enzymes in the studies of Bacilli) is absent from the literature. Principal deterrents in the pursuit of such information have been, and still are, the special techniques required for obtaining and maintaining anaerobiosis, the difficulties of synchronizing growth and sporulation, and the risk in handling those clostridial cultures which produce powerful neurotoxins.

Toxin Formation

The neurotoxins of Clostridium botulinum are intracellularly produced proteins which appear in the surrounding substrate primarily upon cell wall deterioration and autolysis. A nontoxic precursor ("prototoxin") has been found in strains of type A, B, E, and F; strains of type C and D have as yet not been tested for this precursor. In the proteolytic strains, the precursor is activated by proteolytic enzymes both intracellularly and concomitant with autolysis (Bonventre and Kempe, 1959a). It is not known

how the "prototoxins" of the nonproteolytic strains of types B, E, and F become toxic. Conceivably, these strains are slightly proteolytic in spite of their general description as being nonproteolytic. This may also explain their usually low toxin titers (Duff, et al., 1956). However, the toxicity of culture filtrates from these strains can be increased 10 to 10,000 times by activation with trypsin or proteolytic enzymes from bacteria and fungi (Duff et al., 1956). Culture filtrates from very young proteolytic strains are also susceptible to activation; however, the toxicity of filtrates from autolyzed cultures can no longer be increased by treatment with proteolytic enzymes (Bonventre and Kempe, 1959b). Although there seems to be some protease specificity in the activation of botulinal toxins (Inukai, 1963), trypsin activation is used routinely by investigators for all strains, in particular the nonproteolytic strains of types B, E, and F.

To date, five of the six types of Clostridium botulinum toxins have been isolated and highly purified; type F toxin has not been purified as yet. Only type A has been obtained in crystalline form (Lamanna et al., 1946; Abram et al., 1946).

Type A toxin was the first bacterial toxin to be crystallized. It was found to be a white and odorless compound and, at the time of discovery, was characterized as a simple homogeneous protein of 900,000 molecular weight (Lamanna et al., 1946; Putnam et al., 1946) containing only amino acids whose composition did not differ greatly from those of many nontoxic proteins. In the meantime, improved analytical techniques have shown that the crystalline type A toxin can be resolved into at least two different proteins, a toxic alpha fraction and a nontoxic,

hemagglutinating beta component (DasGupta and Boroff, 1968; DasGupta et al., 1966). The alpha fraction is free of hemagglutinating activity and its specific toxicity is five times that of the crystalline toxin. Determinations of the molecular weight of the alpha fraction range from 128,000 (Boroff et al., 1966) to 150,000 (DasGupta and Boroff, 1968) and 158,000 (Wagman, 1963) depending on the method of fractionation and purification. Studies on the molecular dimensions of the alpha component by gel filtration show it to be 9.6 nanometers in diameter, a value which corresponds well with that of toxin found in body fluids and cholinergic junctions. It has been suggested, therefore, that the toxic alpha fraction is capable of penetrating the intestinal wall and reaching its specific site of action. This possibility would eliminate the difficulties encountered in understanding how a macromolecule of 900,000 molecular weight is able to pass through the intestinal barrier and enter the blood stream.

Recent studies on type E toxin have given considerable support to the idea that the toxic macromolecule may dissociate into at least one toxic fraction (alpha) and one nontoxic fraction (beta). Kitamura et al., (1969; 1968) succeeded in purifying type E toxin as well as its trypsin-activated form. Each toxin was found to have the same molecular size of $S^{\circ}_{20,w} = 11.6$ and the same molecular weight of 350,000. When exposed to gel filtration on Sephadex G-200 at pH 8 both toxins dissociated into a toxic E-alpha and a nontoxic E-beta component, each with a molecular weight of 150,000.

In an effort to elucidate the pathogenic mechanism of the type E macromolecule, Kitamura et al., (1969) tested the stability of the undissociated toxins, the parental as well as active form, and their dissociated

alpha fractions at different temperatures and pH values. It was found that the dissociated toxic components were much more unstable than the undissociated macromolecules, losing their toxicity more rapidly at increasing temperatures and decreasing pH values. The difference in stabilities was particularly pronounced below pH 4. These results suggested to the investigators that the E-beta component may function as a stabilizer of the E-alpha fraction. Thus, the undissociated type E toxin molecule may pass through the stomach without appreciable loss of toxicity, whereas the low pH of the gastric juice would rapidly detoxify the alpha component if it were ingested by itself. Studies have indicated that type E (Sakaguchi et al., 1966) as well as type A toxin (Schantz and Spero, 1967) are present in contaminated foods as undissociated macromolecules. These may pass through the stomach into the intestine where at least the type E toxin may become trypsin-activated. In the more alkaline regions of the intestine the macromolecules may then dissociate, and the toxic alpha fractions be absorbed into the lymph and the blood stream. Indeed, the toxic substance found by Kitamura and co-workers in the blood of test animals, which had been given type E toxin orally, was identified as the E-alpha fraction, analagous to the findings of DasGupta and Boroff (1968) in the case of type A crystalline toxin.

The 150,000 molecular weight toxic alpha fractions have so far been discovered in type A, type B (DasGupta et al., 1968), and type E toxin and are presumed to be present also in the remaining types (Kitamura et al., 1969). However, the unique biological activities of these toxins has yet to be determined. Schantz and Spero (1957) showed that cysteine is not involved in the toxicity. Boroff et al., (1966) noted that the

toxin fluoresces at a wavelength characteristic of tryptophan (350 nanometer). Subsequent studies revealed that upon photo-oxidation the toxic alpha fraction loses its fluorescence and toxicity, and that the loss is related to the length of exposure to light. It was also observed that the photo-oxidized alpha component fails to react with antiserum to crystalline toxin. These results lent support to the hypothesis that some tryptophan residues are vital to the maintenance of reactive sites responsible for toxicity and formation of specific antibodies which neutralize the toxin.

The 150,000 molecular weight toxic alpha proteins may be fractionated into smaller subunits with molecular weights ranging from 9,000 to 12,000. These moieties have also been found to be toxic (Gerwing et al., 1965-type A; Wagman, 1963-type A; Gerwing et al., 1966-type B; Gerwing et al.,1964-type E). Their toxic activity, as measured per milligram of nitrogen, is similar to that of the parental type toxins. However, these low molecular weight subunits have not yet been found to exist naturally (Sakaguchi et al., 1966; Schantz and Spero, 1967).

Synchronous Growth

Many aspects of cellular growth and multiplication cannot be studied by using conventional cultures; instead, a high degree of "phasing" or "synchronization" of cells is required. At this point, a distinction between division and cycle oriented synchrony can be made. Depending on how strongly an investigator assumes growth and cell division to be connected, he may argue that division synchrony is the result of cycle synchrony and vice versa. His personal philosophy may well decide where to place the emphasis.

Abbo and Pardee (1960) stressed the difference in the meaning of the words "synchronous" and "synchronized", and Maaløe (1962) distinguished between two categories of synchronization experiments: (1) experiments of the selection type, and (2) experiments inducing synchrony by chemical and physical means. Scherbaum (1964) called cultures resulting from the former type "synchronous cultures" and those from the latter "synchronized cultures".

Synchronized Cultures. The first efforts to synchronize cell populations concentrated on chemical and physical induction techniques which were successful in producing artificial synchrony of division. Changes in environmental conditions such as temperature, light, and nutrition have been used as inducing agents (James, 1966). None, however, can be said to be so specific in its action as to induce or inhibit a particular event in the cell cycle that in turn will trigger or inhibit the mechanism for cell division. Cold shocking, for example, will most often inhibit or delay DNA replication which is then reinitiated upon exposure of the cells to a warm phase. It can hardly be expected, though, that the effect of the temperature change is confined to one specific cellular component. The "specificity" of a synchronizing agent must also be evaluated against the type of cell that is used in an experiment. Some agent may be specific for procaryotic cells but may prove ineffective for eucaryotic systems due to the wide spectrum of cellular complexities inherent in these two cell types.

One of the most specific methods in controlling cell division has been the use of thymine starvation of a thymine-requiring strain of *E. coli* 15T⁻ (Barner and Cohen, 1956). When a culture of this strain is

transferred from an optimum medium to a thymine-free medium, DNA synthesis and cell division are blocked immediately, but RNA and protein synthesis are unaffected. When thymine is added to the medium, after an optimum of 30 minutes of starvation, most cells divide synchronously following a lag of 30-40 minutes. From this thymine-less strain further auxotrophic mutants were isolated which required arginine and uracil or tryptophan and proline for RNA and protein synthesis. By selectively inhibiting either DNA, or RNA and protein synthesis, in these triple mutants, it was found that DNA replication can be completed in the absence of RNA and protein synthesis, but a new round of DNA synthesis can no longer be initiated unless the cell is able to increase its mass in proportion to the increase in DNA. This mechanism by which cells control their DNA/mass ratio has also been observed to be affected by single temperature shifts (Maaløe, 1962; Hanawalt, 1961).

The findings of experiments with artifically induced division synchrony have lent support to the hypothesis that growth and cell division are separate but interdependent processes - shifts in temperature and nutrition or a combination of both cause a unified response of RNA and protein (mass) synthesis on the one hand and DNA and cell division on the other, but neither group acts without some control from the other. The mechanism(s) of interdependence has yet to be determined.

Synchronous Cultures. The term "artificial" synchrony implies the existence of "natural" synchrony. There are many naturally synchronizing systems in nature such as marine eggs in which cleavages occur simultaneously. In a few instances synchronous division is also observed in natural populations of protozoa (Campbell, 1957). However, for bacterial

cultures synchrony is not known to occur spontaneously. Nevertheless, under optimum conditions bacterial cells may exhibit a certain property which bacteriologists have utilized in defining "normal" growth. This property is exponential or steady state growth (Davis et al., 1968). In dilute liquid media the number of bacterial cells tend to increase exponentially with respect to time and the average chemical composition stays constant. This mode of growth can be maintained as long as one wishes provided that appropriate dilutions are made or, in thermodynamic terms, a large capacity source and sink are available. Such growth in a chemostat is said to be balanced and assumes that all cell components double in quantity from one cell division to the next. Under these conditions, a "normal" division cycle may be defined as the sequence of events which lead to the doubling of all cell components (Maaløe, 1962).

In the laboratory, such normal growth may be synchronized by selection techniques which minimize metabolic disturbances. The basis for these methods is the isolation of a specific physical or physiological characteristic which may exist only momentarily at some time during the cell cycle. The resulting selection synchrony, as opposed to the induction synchrony described previously, can be used to study patterns of enzyme synthesis, the mapping of genetic loci responsible for enzyme and protein synthesis, inducibility of enzymes at particular stages in the cell cycle, and the correlation between synthesis and the appearance of structural components, as in the case of spore formation. Furthermore, an insight into the regulatory mechanisms of growth and division may be gained by comparing the events in an unperturbed synchronous culture with the events in a culture whose synchronous growth has been deliberately imbalanced by chemical or physical stresses.

Three techniques have been successful in producing selection synchrony in some bacterial populations. Mitchison and Vincent (1966) developed a rapid method of selecting a homogeneous fraction of cells by density gradient centrifugation. Cells from the exponential phase of growth are concentrated and layered on top of a linear sucrose gradient and then centrifuged. The smallest cells, still at the beginning of the cell cycle, can be taken off the upper portion of the gradient and subcultured in fresh medium, where they develop as a synchronous culture. Metabolic disturbance of the cells is minimized if the gradient is made up in a growth medium. Should the sucrose cause osmotic injury to the cells, the gradient may also be made with other macromolecules such as dextran or protein.

A second method was developed by Helmstetter and Cummings (1964; 1963) while exploring possible improvements to the filtration technique of Maruyama and Yanagita (1956). The latter investigators filtered their cultures through stacks of filter paper assuming that the organisms played a passive role during filtration and that synchronous growth was a consequence of selection by size. Helmstetter and Cummings discovered, however, that bacteria will adsorb to a variety of membrane filters and will then proceed to divide when exposed to a constantly changing supply of medium. Thus, by binding cells to a membrane surface, inverting the membrane, and subjecting the adsorbed organisms to a constant flow of medium, newly formed daughter cells are eluted. These produce synchronous cultures when subcultured in fresh medium.

A third technique allows asynchronous populations of cells to grow until a particular time in the stationary phase is reached. The cells are

then harvested and subcultured in fresh medium at about a seven-fold dilution. The optimal harvest time appears to vary among species and repeated subculturing may also be necessary to improve the synchrony obtained (Cutler and Evans, 1967; Masters et al., 1964; Yoshikawa et al., 1964).

Finally, a unique method of achieving division synchrony in spore forming bacteria utilizes the process of spore germination. The transformation of a dormant spore into a vegetative cells is known to occur in three sequential steps: activation, germination, and outgrowth (Keynan and Halvorson, 1965). These processes are fundamentally different from each other. Activation conditions the dormant spore to germinate if the environment is conducive to germination. Accumulating evidence suggests that activation does not involve active metabolism but rather reversible changes in the configuration of macromolecules (Keynan and Evenchick, 1969). The activated spore retains most of its spore properties.

Germination, once initiated, is irreversible and results in a complete loss of typical spore characteristics such as metabolic inactivity, refractility, resistance to heat, irradiation, and chemical agents. Yet, the cell is still quite distinct from its vegetative counterpart. The process of germination does not involve the synthesis of new macromolecules but rather the degradation of spore components: the spore swells, breaks out of its coats, and excretes up to 30 percent of its dry weight in the form of mucopeptides and calcium chelates of dipicolinic acid - all spore specific substances (Powell and Strange, 1953).

In a complete medium, outgrowth follows as a period of sudden biosynthetic activity during which new kinds of proteins are formed that did not pre-exist in the dormant spore (Kobayshi et al., 1965). The simplest

method of activating spores is exposure to sublethal heat. Under the proper nutritional conditions very rapid germination and outgrowth follow, leading to a culture which divides synchronously for several generations (Kobayashi et al., 1965; Wake, 1963; Young and Fitz-James, 1959).

Little use has been made of the available synchronizing methods with regard to sporulation. Most studies on sporulation have utilized the conventional batch culture technique starting with a heavy spore inoculum. The ensuing growth exhibits the same patterns that are typical of nonsporulating procaryotes, namely lag, logarithmic, and stationary phases. Sporulation commences during the transition from the late exponential phase of growth to the stationary phase, but synchrony of growth and sporulation does not occur. However, a modification of the batch culture method, the so-called "active culture" technique (Halvorson, 1957), has been successful in synchronizing at least the late stages of sporulation in Clostridium roseum, as shown by the rapid appearance of refractile (stage IV - Murrell, 1967) and heat resistant (stages V - VI) spores. Basically, the method consists of building up a culture from an initial spore inoculum. The spores are induced to germinate and a ten percent (by volume) inoculum is transferred to fresh medium just as the vegetative cells prepare to sporulate again. This subculturing is repeated a total of three times. Then a final inoculum, again ten percent by volume, is made into fresh sporulation medium whose chemical composition differs from that of the germination medium. "Rapid and simultaneous" spore formation commences within a few hours. Although this method has been valuable in defining many events during the late stages of spore formation, it has not been explored in the literature for possible synchrony of the vegetative

growth in order to obtain information on the induction and regulation of sporulation.

Imanaka et al. (1967) obtained synchronous growth and sporulation of Bacillus megaterium by filtering late log phase batch cultures through nine layers of Whatman No. 40 filter paper in a fritted-glass Büchner funnel. Cells, when grown in a defined sucrose-salt medium (SS) go through one synchronous division after filtration and then proceed to sporulate; cells grown in SS-medium supplemented with glutamate complete two synchronous divisions before sporulating. Although this method claims good division and sporulation synchrony, there have been no further reports in the literature which examine the sequential events during sporulation of Bacillus megaterium and possible induction and regulation mechanisms.

The preceding summary of present information on spore and toxin formation and synchrony in Clostridium botulinum points out that a considerable amount of basic knowledge is still to be established for type F, and that studies on the sequential biochemical events of spore and toxin formation are almost non-existent for any of the botulinal types.

In developing the idea for this thesis, it seemed to me that synchronizing the biochemical events which lead to the production of a spore or toxin molecule, and subjecting the sequence to qualitative and quantitative analyses, would provide an investigator with a good method to gain a refined understanding of spore and toxin formation. If a reproducible pattern of synthesis can be established, it should then also be possible to interfere with that pattern by introducing chemical or physical changes in the environment of the cell population. Hopefully, the reaction of the cells to such environmental stresses will yield valuable information not

only on the induction mechanism(s) of spore formation but also on possible means of interfering with the formation processes of spores and, in particular toxin. It is hoped that useful applications for the various food industries can be derived from such extended knowledge of spore and toxin production. Establishing a synchronizing technique for Clostridium botulinum, type F, strain Langeland would also have the immediate advantage of providing a basis for continuous research beyond the present level of a master's degree.

CHAPTER II

MATERIALS AND METHODS

Organism Used

Clostridium botulinum, type F, strain Langeland was the test organism used throughout this study. The organism was obtained from Dr. C. E. Dolman of the University of British Columbia, Canada, and maintained as a stock culture in the laboratories of Dr. N. W. Walls, Engineering Experiment Station, Georgia Institute of Technology, Atlanta, Georgia. Unless otherwise stated, the incubation temperature used throughout this investigation was 30 C.

Toxin Characterization

The organism obtained from Dr. Walls, was subjected to both biochemical and toxin characterization tests in order to confirm its identity and purity. The culture was inoculated into 30 ml of Cooked Meat Medium (Difco) in a 25 x 200 mm screw cap tube and incubated at 30 C until spores were produced as determined by microscopic examination (see Appendix A for spore staining procedure). An aliquant of the culture was then transferred into a sterile 16 x 125 mm screw cap tube and heat shocked at 85 C for 15 minutes to destroy all vegetative cells. A ten percent (by volume) inoculum from the heat shocked sample was then added to 30 ml of freshly prepared cooked meat medium and incubated for 24 hours. A smear of the culture was Gram stained (see Appendix A) to check for obvious contamination. Dry Liver-Veal Agar (BBL) plates containing 0.5 percent (w/v)

vegetable lecithin were streaked with the culture and incubated in an anaerobic "Gaspak" jar (BBL). To prevent the spreading of colonies due to the accumulation of moisture in the "Gaspak" jars, sterile Whatman filter pads containing a drop or two of sterile glycerol were placed inside the covers of the agar plates. Streaked plates were also incubated in an aerobic atmosphere. After 48-72 hours growth was luxuriant on the anaerobically incubated plates; there was no growth on the aerobically incubated plates. Single opalescent colonies, convex, irregular in shape, and surrounded by a pearly zone on the surface of the agar and a large zone of opaque precipitate in the agar were picked and inoculated into freshly prepared cooked meat medium. After five days of incubation the cultures were tested for toxicity in the following manner:

Aliquots were spun at 3000 x g for 30 minutes in a refrigerated centrifuge* at 4 C to clear the culture fluid of all particulate matter. For each culture, ten mice (Swiss White strain, male, 13-18 grams in weight) were injected intraperitoneally with undiluted, unboiled supernatant liquid and another ten mice with supernate which had been heated in a boiling water bath for ten minutes. The dose for each mouse was 0.5 ml. The animals were observed for typical symptoms of botulism and for death; survivors were sacrificed after 4 days.

Those cultures which produced death in the unboiled but not in the boiled supernate were then tested for the specific immunological type of botulism involved. The toxic supernatant liquids were mixed with the

*Model PR-2; International Equipment Co., Needham Hts., Mass.

antitoxins of types A, B, C, D, E, or F so that each 0.5 ml contained one International Unit (IU) of antitoxin (NCDC, 1964). At the same time, mice were injected again with boiled or unboiled culture supernate free of antitoxins. The results of toxicity testing and typing are presented in Table 1.

One of the pure cultures, against which type F antitoxin had provided complete protection, was now used for further biochemical characterization and the production of a large number of frozen stock cultures as described in the section on "Maintenance of Stock Cultures".

Biochemical Studies

The ability of the organism to ferment certain carbohydrates was tested in a basal medium containing 1.0 percent Trypticase (BBL), 1.0 percent Proteose Peptone (Difco), and 0.1 percent Sodium Thioglycollate (Difco). Filter sterile carbohydrate solutions were added to a final concentration of 1.0 percent just prior to inoculation. Bromthymol blue indicator was added to aliquots of the culture after incubation for 1, 3, 5, and 7 days.

The ability to digest coagulated egg albumin was observed in Trypticase Soy Broth (BBL) to which egg white had been added before autoclaving (one egg white per 1000 ml of TSB).

Other biochemical characteristics were determined using the composition of media and evaluation of reactions as described by Haldeman Holdeman (1964). The results are presented in Table 2.

Maintenance of Stock Cultures

The known strains of Clostridium botulinum have been found to be unusually mutagenic (Dolman and Murakami, 1961). In order to minimize the

Table 1. Neutralization Pattern of *Clostridium botulinum*, Type F, Strain Langeland.

SOURCE OF TOXIN	ANTITOXIN (1 IU/DOSE	NUMBER OF MICE DEAD	ALIVE
Supernate	A	10	0
"	B	10	0
"	C	10	0
"	D	10	0
"	E	10	0
"	F	0	10
" (Heated)	None	0	10
"	None	10	0

Table 2. Cultural and Biochemical Properties of Clostridium botulinum Type F, Strain Langeland.

TESTS	REACTIONS	TESTS	REACTIONS
SACCHAROLYTIC		PROTEOLYTIC	
Dextrose	AG +	Iron Milk	Black, Digested
D-Fructose	AG	Cooked Meat	Black, Digested
Maltose	AG	Gelatin	Digested
D-Trehalose	AG	Coagulated Egg	
Starch	AG +	Albumin	Digested
Dextrin	AG		
Salicin	AG	OTHER	
Glycerol	AG +	Motility	+
D-Sorbitol	AG	Gram's Stain	+
Sucrose	-	Spore Stain	+
Lactose	-	Beta Hemolysis	+
Xylose	-	H_2S Production	+
Melibiose	-	Lecithinase	+
Raffinose	-	Catalase	-
D-Galactose	-	Urease	-
D-(-)Ribose	-	Nitrate Reduction	-
D-(-)Arabinose	-	Indole Production	-
L-(+)Arabinose	-		
Mannitol	-		
Dulcitol	-		
Inositol	-		

Symbols: AG = Acid and Gas
AG+ = Acid and one quarter of liquid medium in fermentation vial replaced by Gas.

influence of this particular characteristic on the results of this investigation, frozen stock cultures of the Langeland strain were prepared as follows:

A pure culture of the organism was incubated in a 25 x 200 mm screw cap culture tube containing 30 ml of cooked meat medium until spores were produced, as determined by microscopic examination. An aliquant of the culture was then transferred to a sterile 16 x 125 mm screw cap tube and heat shocked at 85 C for 15 minutes to destroy vegetative cells. A ten percent (by volume) inoculum from the heat shocked sample was then added to 30 ml of freshly prepared cooked meat medium and incubated for 3 days. At the end of that time, all of the culture supernatant liquid (about 19 ml) was inoculated into a 300 ml volume of cooked meat medium contained in a 500 ml screw cap Erlenmeyer flask. After 5 days of incubation, the supernate was dispensed in 2.5 ml quantities into sterile storage vials which were sealed with paraffin coated corks and quick frozen at -80 C in a 2-ethoxyethanol-dry ice bath. These stock cultures were properly labelled, stored in a freezer at -20 C, and used to initiate each new experiment.

Preparation of Spore Crops

The production of a spore crop was initiated by thawing a tube of frozen stock culture in cold water and transferring its contents to 30 ml of freshly prepared cooked meat medium. After 3 days incubation, all of the culture supernatant liquid was added aseptically to 300 ml of cooked meat medium in a 500 ml Erlenmeyer flask and incubated for 5 days. The supernate from this culture was then transferred into 2000 ml of Type C Toxin Medium, the final sporulation medium (see Appendix A for formula). Production

of spores within the culture was followed by microscopic examination of stained smears (see Appendix A) made at frequent intervals. The culture was judged ready for harvesting when no further increase in the percentage of spores produced was observed. This usually occurred in 10-15 days.

Collection of Spores

When spore production had reached its peak, 200 ml quantities of the culture were poured into each of six sterile 250 ml centrifuge bottles. These were placed in sealed centrifuge cups and spun in a refrigerated centrifuge (IEC Model PR-2) at 2300 x g for 30 minutes at 4 C. The supernatant liquid was decanted and discarded after autoclaving. The sediment, containing spores, vegetative cells and debris, was resuspended and washed in 200 ml of cold, sterile, deionized water a minimum of three times, spinning after each washing and discarding the supernate.

The sediments were pooled and redistributed among four and then two centrifuge bottles, each time repeating the washing procedure at least twice. The last wash water was carefully siphoned off and the spore pellet resuspended in as small a volume of cold, sterile, deionized water as possible. Finally, the spore suspension was aseptically transferred to a 50 ml sterile screw cap Erlenmeyer flask containing a few glass beads. The flask was labelled with the name of the organism, sporulation medium, and date, and refrigerated at 4 C. The stock suspension was titrated for viable numbers of spores as described in the section on "Counting Methods".

Cleaning of Spores

Whenever an experiment was initiated with an aliquot of the spore

suspension it was necessary to clean all cellular debris from the sample to avoid increasing the error in the optical density readings and total counts and to eliminate the possibility of obstructing the light path through the flowcell of the Technicon Auto-Analyzer colorimeter which was used to monitor the growth of a culture.

The cleaning procedure adopted was developed by Mr. G. R. Bell (Georgia Institute of Technology, Engineering Experiment Station) as a modification of Grecz's method (Grecz, et al., 1962). The procedure alternates sonication with digestion by trypsin and lysozyme at 45 C (see Appendix A).

Equipment

The optical density of a growing culture was measured and recorded continuously at a wavelength of 520 nanometers by an Auto-Analyzer assembly* which consisted of a proportioning pump, colorimeter, and recorder. Although the general operating instructions for each instrument were followed closely, a few modifications were devised in order to adapt the instrument assembly for the continuous recording of bacterial growth curves.

The cultures were grown in suitable vessels which were held firmly in place by a clamp in a 30 C water bath.** The vessels had openings to allow for: (i) the withdrawal of samples at specific intervals for total and viable counts, (ii) continuous bubbling of nitrogen through the medium by means of a section of standard glass tubing slightly bent and extending to near the bottom of each vessel, and (iii) 4 mm (O.D.) withdrawal and return

*Technicon Instruments Corp., Chauncey, New York.

**Model 220; National Appliance Co., Portland, Oregon.

glass tubing to which the plastic transmission tubings of the Auto-Analyzer assembly were attached, allowing easy separation of a culture vessel from the Auto-Analyzer after the completion of an experiment. The glass withdrawal tubing extended approximately half-way into the medium whereas the return tubing only about one half inch below the surface. The continuous bubbling of nitrogen helped maintain anaerobiosis and a fairly even distribution of organisms in the culture medium. Even distribution was also encouraged by continuous stirring with a magnetic stirring bar. All culture vessels were placed on a flat, round magnetic stirrer* whose turbine rotor was water-driven by a small immersion pump**. This combination also served to circulate the water in the bath to maintain a constant incubation temperature.

The continuous and constant withdrawal from, and return of, medium to a culture vessel was assured by using green coded pump tubing with a nominal delivery rate of 2 ml per minute in the proportioning pump. Two debubblers were installed in the withdrawal tubing leading to the flowcell: the first one (a modified C-5 debubbler) between the proportioning pump and flowcell, and the second (a regular C-5 debubbler) at the inlet to the flowcell. This particular arrangement was found necessary to remove the large quantities of gas evolved during the decelerating log-phase of culture growth. A flow diagram is shown in Figure 1. One arm of each debubbler was connected to a waste Erlenmeyer flask with standard plastic tubing (1/8" I.D. x 1/4" O.D.). The flask was fitted with a rubber stopper with holes for three lengths of 4 mm (O.D.) glass tubing, one serving as a vent, the

*Chemical Rubber Co., Cleveland, Ohio.

**Gorman Rupp Industries, Inc., Bellville, Ohio.

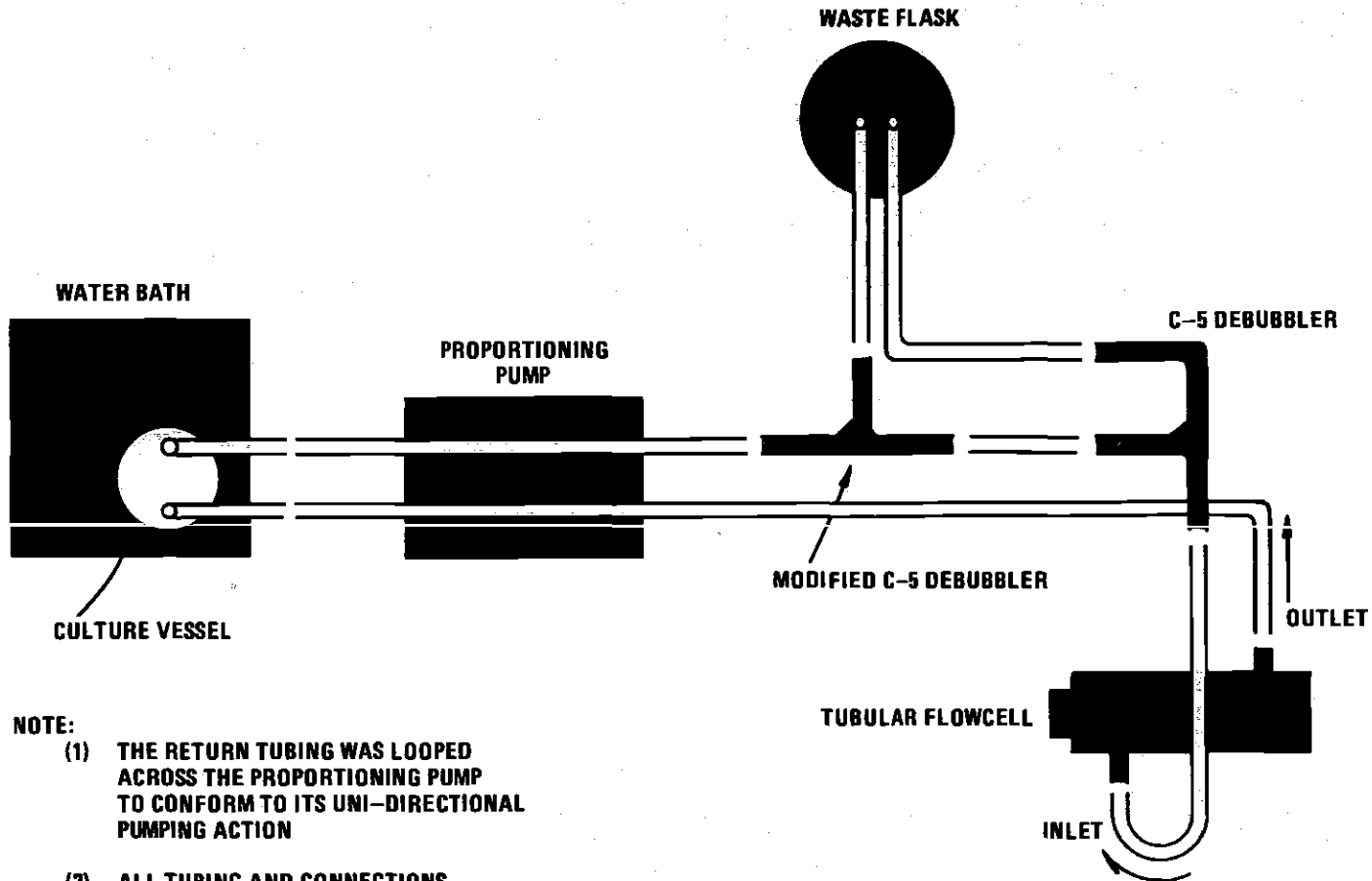

Figure 1. Flow Diagram for the Continuous Recording of the Growth of Clostridium botulinum, Type F, Strain Langeland Using a Technicon Auto-Analyzer Assembly.

other two connected to the waste tubing. Thus, the flask could be removed easily for autoclaving of the waste liquid. The glass ends of each waste line were sealed with a Bunsen valve so that bubbles and waste fluid were able to escape into the flask, but air was prevented from entering the continuous flow of the culture medium.

The water bath, proportioning pump, and colorimeter were spaced as closely together as possible so that a sample was out of the culture vessel no longer than two minutes. The arrangement of the instrument assembly is presented in Figure 2. It includes a Coulter Counter* with a 100 micrometer aperture used in the determination of total counts.

Counting Methods

Viable Counts

In order to determine the number of viable cells in a growing culture, or the viable spores in a stock suspension, appropriate 100-fold serial dilutions were made of a 1.0 ml sample using sterile peptone water contained in milk dilution bottles (see Appendix A). In the case of the spore sample, heat shocking at 85 C for 15 minutes prior to serial dilution was necessary in order to destroy all vegetative cells. After shaking each dilution bottle vigorously to insure a homogeneous distribution of the cell or spore sample, correct aliquots were pipetted aseptically into Prickett tubes (modified Fisher agar slant tubes) containing fresh pork infusion medium (see Appendix A) held at 45 C in a water bath. Each dilution was done in triplicate. The tubes were quickly rolled between the palms of the hands and plunged into an iced water bath to solidify

*Model B; Coulter Electronics Industrial Division, Hialeah, Florida.

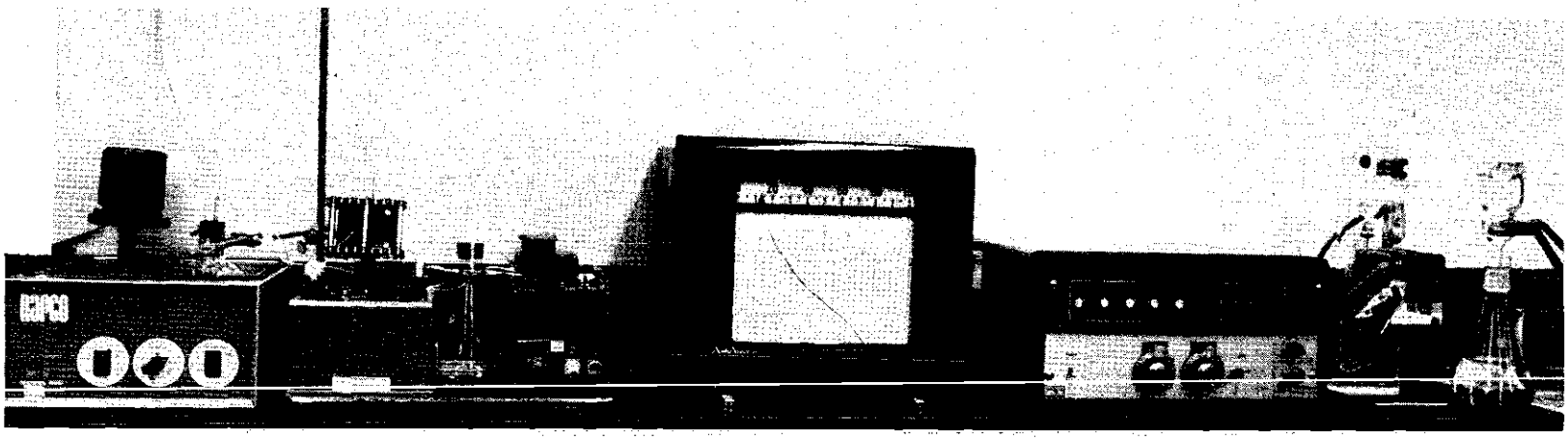

Figure 2. Arrangement of Technicon Auto-Analyzer Assembly and Accessories for the Continuous Recording of Growth Curves of Clostridium botulinum, Type F, Strain Langeland. (A) Water Bath With Immersion Pump and Culture Vessel; (B) Technicon Proportioning Pump; (C) Waste Flask; (D) Technicon Auto-Analyzer Colorimeter; (E) Technicon Auto-Analyzer Recorder; (F) Coulter Counter Model B; (G) Millipore Filter.

the recovery medium quickly and so accomplish an even distribution of the bacterial cells throughout each tube. Upon solidification, each tube was sealed with sterile two percent Bacto-Agar (Difco) containing 0.1 percent sodium thioglycollate, and the cotton plug replaced. The tubes were incubated for 5-7 days before the colonies were counted under a 1-1/2 X magnifying glass of a Bactronic Colony Counter*.

The arithmetic mean and its standard deviation were calculated from the triplicate counts of each dilution. If the standard deviation exceeded 20 percent of the value for the mean, the count with the largest deviation from the mean was eliminated. Counts of less than 10 colonies per tube were within the limits of titration and dilution errors and therefore not acceptable; counts of more than 200 colonies were also rejected because they were subject to a large counting error due to the restricted size and geometry of the Prickett tubes.

Total Counts

At regular intervals, a 1.0 ml sample was withdrawn from a growing culture, diluted in a milk dilution bottle containing 99 ml of physiological saline (0.85 percent sodium chloride), and shaken vigorously in order to achieve a homogeneous distribution of organisms. About 40 ml of this suspension were used for total count determinations with the Coulter Counter.

Calibration of Coulter Counter

Preliminary sizing experiments had shown no significant numbers of organisms greater than 3.5 micrometers in size, and those less than 1.0 micrometer could not be distinguished from the electronic background noise.

*Model CC-110; New Brunswick Scientific Co., New Brunswick, New Jersey.

This left a range of 1.0-3.5 micrometers to be considered for counting. The range was subdivided into suitable intervals as shown in Table 3. The corresponding values for the lower and upper threshold settings and multipliers (1/amplification and 1/aperture current) were taken from data supplied by Coulter Electronics.

The accuracy of the instrument settings was checked by determining the size distribution of polyvinyltoluene latex microspheres* labelled as 1.011 ± 0.005 μ, 2.051 ± 0.0017 μ, and 3.49 ± 0.0018 μ in size. The corresponding instrument settings are summarized in Table 4. The total counts were not corrected for coincidence.

Preparation of Electrolyte

The counting limit of the Coulter Counter of 1,000,000 particles per ml required appropriate dilutions of the culture samples. An 0.85 percent solution of sodium chloride was used as the electrically conductive diluent. The saline solution was filtered through a 0.45 micrometer Millipore filter** just prior to use, and 99 ml quantities were measured into milk dilution bottles. This procedure assured a total background count of less than 2000 particles per ml.

When a filter was replaced, a 300 ml quantity of the electrolyte was filtered through the new one in order to dislodge all fine particulate matter which would significantly raise the background count. The filtrate was discarded and the receiving vessel thoroughly rinsed with distilled water.

Care of Aperture

Before the start of an experiment the glass assembly containing the

*Dow Chemical Co., Midland, Michigan.
**Millipore Filter Corp., Bedford, Massachusetts.

Table 3. Coulter Counter Instrument Settings for Total Count Determination of Clostridium botulinum, Type F, Strain Langeland.

Instrument Settings				Equivalent Particle Sizes	
Multipliers		Thresholds		Range	Mean Diameter
A*	B**	Lower	Upper	(Micrometer)	
1/4	1/4	5	10	1.0 - 1.5	1.25
1/4	1/4	10	23	1.5 - 2.0	1.75
1/4	1/4	23	41	2.0 - 2.5	2.25
1/4	1	10	20	2.5 - 3.0	2.75
1/4	1	20	30	3.0 - 3.5	3.25

*A = 1/ amplification
**B = 1/aperture current

Table 4. Coulter Counter Instrument Settings for Determining Size Distributions of Polyvinyltoluene Latex Microspheres.

Instrument Settings				Equivalent Particle Sizes	
Multipliers		Thresholds		Range	Mean Diameter
A*	B**	Lower	Upper	(Micrometer)	
1. For Latex Microspheres Labelled 1.011 ± 0.005 μ					
1/8	1/8	4	6	0.5-0.7	0.6
1/8	1/8	5	8	0.6-0.8	0.7
1/8	1/8	6	10	0.7-0.9	0.8
1/8	1/8	8	13	0.8-1.0	0.9
1/8	1/8	10	16	0.9-1.1	1.0
1/8	1/8	13	20	1.0-1.2	1.1
1/8	1/8	16	25	1.1-1.3	1.2
1/8	1/8	20	30	1.2-1.4	1.3
2. For Latex Microspheres Labelled 2.51 ± 0.018 μ					
1/8	1/8	40	65	1.5-1.7	1.6
1/8	1/8	50	70	1.6-1.8	1.7
1/8	1/8	65	80	1.7-1.9	1.8
1/8	1/8	70	90	1.8-2.0	1.9
1/8	1/8	80	100	1.9-2.1	2.0
1/4	1/4	23	30	2.0-2.2	2.1
1/4	1/4	26	34	2.1-2.3	2.2
1/4	1/4	30	38	2.2-2.4	2.3
3. For Latex Microspheres Labelled 3.49 ± 0.017 μ					
1/4	1	21	25	3.1-3.3	3.2
1/4	1	23	27	3.2-3.4	3.3
1/4	1	25	30	3.3-3.5	3.4
1/4	1	27	32	3.4-3.6	3.5
1/4	1	30	35	3.5-3.7	3.6
1/4	1	32	38	3.6-3.8	3.7
1/4	1	35	41	3.7-3.9	3.8

*A = 1/amplification
**B = 1/aperture current

100 micrometer aperture was disconnected and cleaned with concentrated nitric acid. After counting a sample, the glass assembly and aperture were cleaned (as pH electrodes are) by rinsing thoroughly with filtered electrolyte from a plastic wash bottle. The sample and rinse were discarded for autoclaving and the sample vessel, a 50 ml plastic beaker, rinsed several times with freshly filtered diluent. These precautions held the total number of particles carried over from one sample reading to the next to less than 10,000 per ml.

Synchronous Growth Experiments

Initiation from Spore Inoculum

A 300 ml quantity of Trypticase Soy Broth (BBL) containing 0.5 percent Yeast Extract (BBL) was prepared with deionized water and sterilized in an autoclave for 20 minutes at 120 C and 15 psig. The medium, contained in a three-neck, 500 ml round bottom flask (Corning #4945), was quickly cooled in iced water until warm to the touch and then immersed in the 30 C water bath with constant stirring as described previously. Anaerobiosis was established by continuous bubbling of nitrogen through the medium and by the aseptic addition of sodium thioglycollate as a 10 percent (w/v) filter sterile solution to a final concentration of 0.1 percent. Medium prepared in this way was used in all synchronous growth experiments and is henceforth designated as TSB^{+}.

When the temperature between water bath and medium had equilibrated, a 1.0 ml sample of cleaned spores (1.0×10^{9} per ml) was used as an inoculum. The spores were heat shocked at 85 C for 15 minutes just prior to use to destroy possible contaminants. A 1.0 ml sample was removed

from the inoculated medium for a "zero hour" viable count and a 2.5 ml sample for the initial optical density reading which, for convenience, was measured in a Spectronic 20 colorimeter* set at a wavelength of 520 nanometers. After removal of the samples, stirring and bubbling of nitrogen were discontinued.

The state of the culture was intermittently checked by carefully withdrawing small samples which were smeared on slides and stained for refractive and germinating spores (see Appendix A). When the spores began to lose their refractivity, stirring and bubbling of nitrogen were resumed and 1.0 ml quantities of culture were removed every 15 minutes for viable counts. The percentage of outgrowth was estimated from stained smears by counting a total of at least one hundred cells and spores and then calculating the respective ratio. As soon as the outgrowth of spores was completed, 1.0 ml samples were also taken for total counts. The optical density of the culture was followed by measuring 2.5 ml samples in the Spectronic 20 colorimeter at the same time that samples were withdrawn for viable and total counts.

After the first doubling of the number of organisms the culture was removed from the water bath and the liquid carefully distributed in two sterile 250 ml centrifuge bottles and spun at 2500 x g (IEC Model PR-2) for 20 minutes at room temperature. The two pellets were each reconstituted with 5.0 ml of fresh medium warmed to 30 C and inoculated into 600 ml of fresh TSB^{+} contained in a three-neck, one liter round bottom flask (Corning #4945). The flask was connected to the Auto-Analyzer

*Bausch & Lomb, Inc., Rochester, New York.

assembly for continuous recording of the culture's optical density. The colorimeter and recorder were prepared for operation according to instructions in the manual*. The recorder was adjusted so that the blank culture medium registered a "zero" optical density on the chart. Viable and total counts were again determined as described above.

Stationary Phase Method

A 2.5 ml sample of frozen stock culture was thawed in cold tap water and inoculated into 50 ml of freshly prepared cooked meat medium in a 125 ml screw cap Erlenmeyer flask. The medium was equilibrated to 30 C prior to inoculation.

After 48 hours of incubation, a loopful of the culture was streaked on plates of Trypticase Soy Agar (BBL) containing 0.5 percent yeast extract and incubated in an anaerobic "Gaspak" jar as described earlier in this paper. An isolated colony was picked after 48-72 hours of growth and inoculated into 20 ml of freshly prepared TSB^{+} in a 20 x 150 mm screw cap culture tube. The size of the tube permitted direct optical density readings of the culture (designated culture "A") in a Spectronic 20 colorimeter set at 520 nanometers.

When a specific stage in the growth of culture "A" was reached, as defined by optical density (see Figure 3), 2.5 ml aliquots were inoculated into each of two 50 ml quantities (about a 20 fold dilution) of fresh TSB^{+} contained in 25 x 200 mm screw-cap tubes. The growth of these cultures (designated cultures "A_1") was followed by removing 2.5 ml samples from one of the culture tubes and measuring the optical density in a Spectronic 20 colorimeter.

*General Operating Instruction Manual, Section C-R; Technicon Instrument Corp., Chauncey, New York.

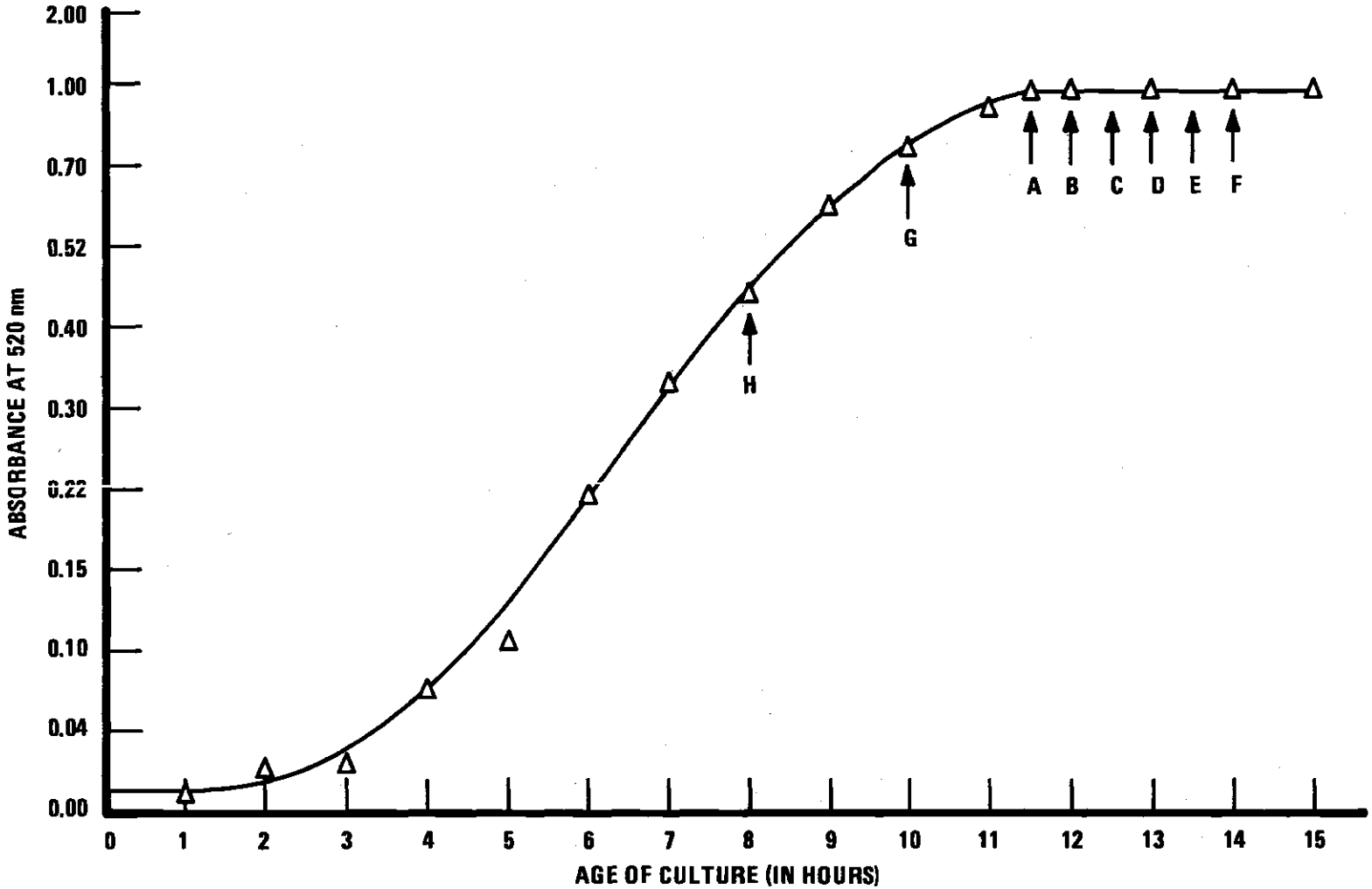

Figure 3. Growth Curve of an Asynchronous Culture of Clostridium botulinum, Type F, Strain Langeland in TSB^{+} at 30 C. Repeated Subcultures Were Made From Selected Points Along the Growth Curve (points A-F: "Stationary Phase Method").

When culture "A_1" attained the same specific optical density as culture "A" had reached previously, all of the culture fluid in the undisturbed tube was transferred to a sterile, 50 ml polypropylene centrifuge tube and spun at 20,000 x g for 5 minutes at room temperature. The pellet was resuspended with 5 ml of fresh medium at 30 C and inoculated into 350 ml of fresh TSB$^+$ (about a 7 fold dilution) in a three-neck, 500 ml round bottom flask held in the water bath at 30 C. The culture (culture "A_2") was continuously stirred as described earlier, and nitrogen was continuously bubbled through the medium in order to maintain anaerobiosis. The growth of the culture was followed by optical density readings and total counts as already described.

The procedure of spinning and resuspending was repeated for a third time, again at the same specific optical density as in the case of the previous cultures, and a final inoculation made into 2500 ml of fresh TSB$^+$ (about a 7 fold dilution) contained in a modified three liter Fernbach flask (Corning #4420). The flask was connected to the Auto-Analyzer for a continuous optical density record of the final culture (culture "A_3") and total and viable counts were determined as described previously.

An identical procedure of subculturing was followed for all other cultures taken from points B through H as indicated in Figure 3.

Further attempts to synchronize Clostridium botulinum, type F by the stationary phase method were made by: (i) slight procedural changes using the TSB$^+$ medium, and (ii) changing to a completely synthetic medium (see Appendix B).

*Model B-20; International Equipment Corp., Needham Heights, Massachusetts.

Cold Shocking Method

A 2.5 ml sample of frozen stock culture was thawed and inoculated into 50 ml of cooked meat medium. After 72 hours of incubation, a 10 percent (by volume) inoculum was transferred to 20 ml of fresh TSB^+ in a 20 x 150 mm screw cap tube and the optical density of the culture followed in a Spectronic 20 colorimeter set at 520 nanometers.

One hour into the stationary phase of growth, as defined by optical density (see Figure 4), 5 ml aliquots were inoculated into each of two 50 ml quantities of fresh TSB^+ contained in 25 x 200 mm screw cap tubes. The growth of these cultures was followed by removing 2.5 ml samples from one of the culture tubes and measuring the optical density in a Spectronic 20 colorimeter.

Again, one hour into the stationary phase of growth, all of the culture fluid in the undisturbed tube was transferred to a sterile, 50 ml polypropylene centrifuge tube and spun at 20,000 x g (IEC Model B-20) for 5 minutes at room temperature. The pellet was resuspended in 5 ml of fresh TSB^+ at 4 C and immediately transferred to a sterile 30 x 120 mm screw cap tube in a refrigerated water bath* set at 4 C. The length of cold shocking was 15, 30, 45, 60, 120, or 180 minutes.

At the end of a selected period of cold shocking, all of the cells were pipetted aseptically into 600 ml of fresh TSB^+ in a three-neck, one liter round bottom flask held in the water bath at a constant temperature of 30 C. The flask was connected to the Auto-Analyzer assembly for a continuous record of the culture's optical density, and total and viable counts were determined as described previously.

*Gilson Omnibath; Gilson Medical Electronics, Middleton, Wisconsin.

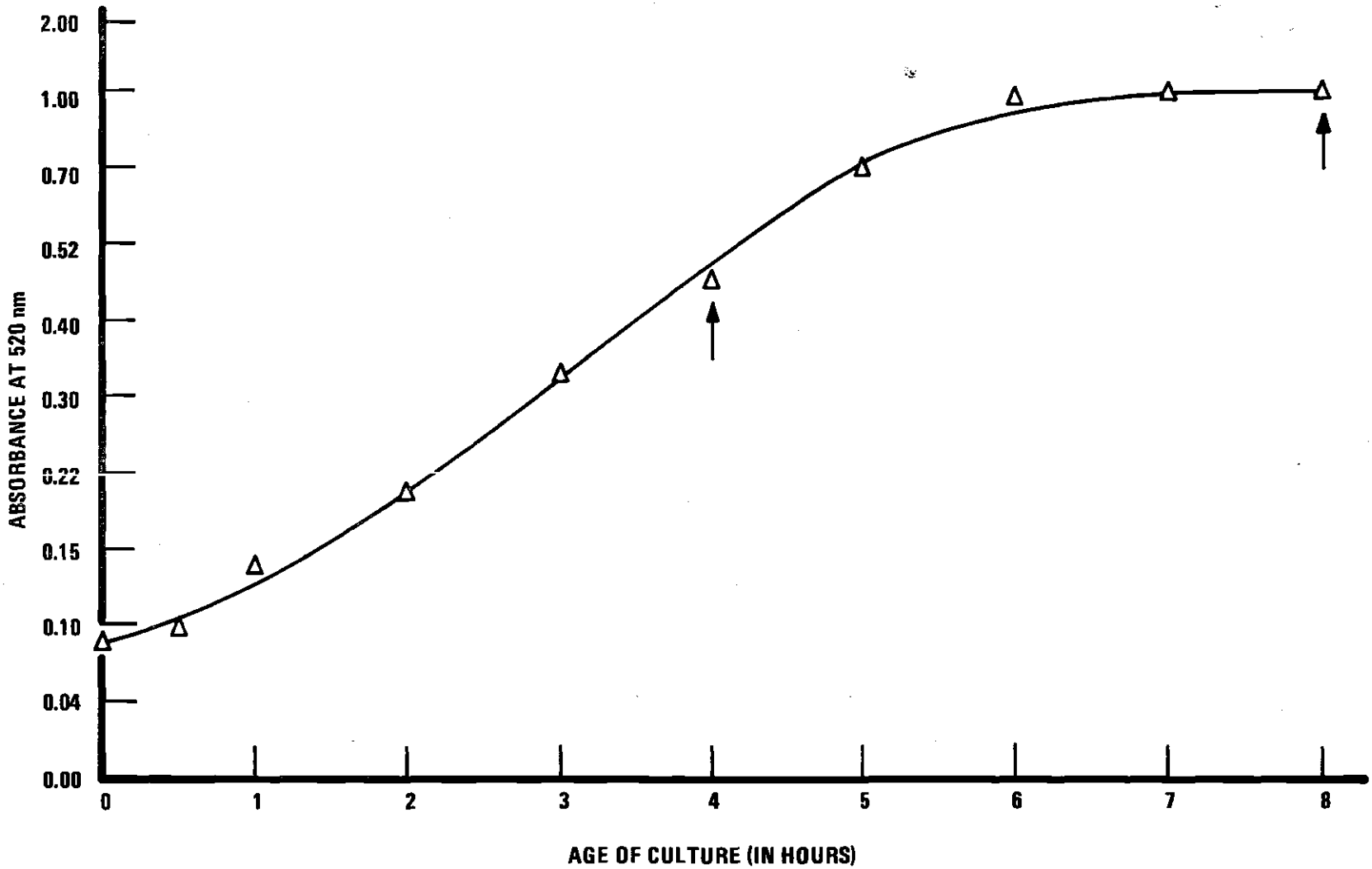

Figure 4. Growth Curve of an Asynchronous Culture of Clostridium botulinum, Type F, Strain Langeland in TSB^{+} at 30 C. Cultures Taken From the Exponential and Stationary Phases of Growth, as Indicated by Arrows, Were Cold Shocked at 4 C for Selected Periods of Time Lasting From 15 to 180 Minutes.

The same procedure was followed for cultures taken from the exponential phase of growth (see Figure 4).

CHAPTER III

RESULTS

Total Count Determinations

The Coulter Counter instrument settings and the equivalent particle sizes were found to be in close agreement as determined from the size distributions of polyvinyltoluene latex microspheres. As seen in Figure 5, most latex spheres labelled by the manufacturer as 1.011 ± 0.005 μ in diameter fell into the 1.0 to 1.2 micrometer range, those labelled as 2.051 ± 0.018 μ into the 1.8 to 2.0 micrometer range, and the ones labelled as 3.49 ± 0.017 μ clustered in the 3.4 to 3.6 micrometer range. Details are presented in Table 5. Although the size distribution curves which may be drawn from the bar graphs in Figure 5 showed a much greater standard deviation (by inspection) than the size descriptions of the latex spheres indicated, the curves did reach their maxima at or near the expected values for the particle diameters. This was thought to be sufficient evidence for the purpose of this study that the instrument settings would give reasonably accurate total cell count determinations.

Synchronous and Synchronized Growth Experiments

Initiation from Spore Inoculum

Spores of Clostridium botulinum, type F, strain Langeland showed signs of germination about 12 hours after inoculation into TSB^{+}. Microscopic examination of spore stains indicated a gradual loss of malachite green uptake. At approximately 13 hours after inoculation more than 90

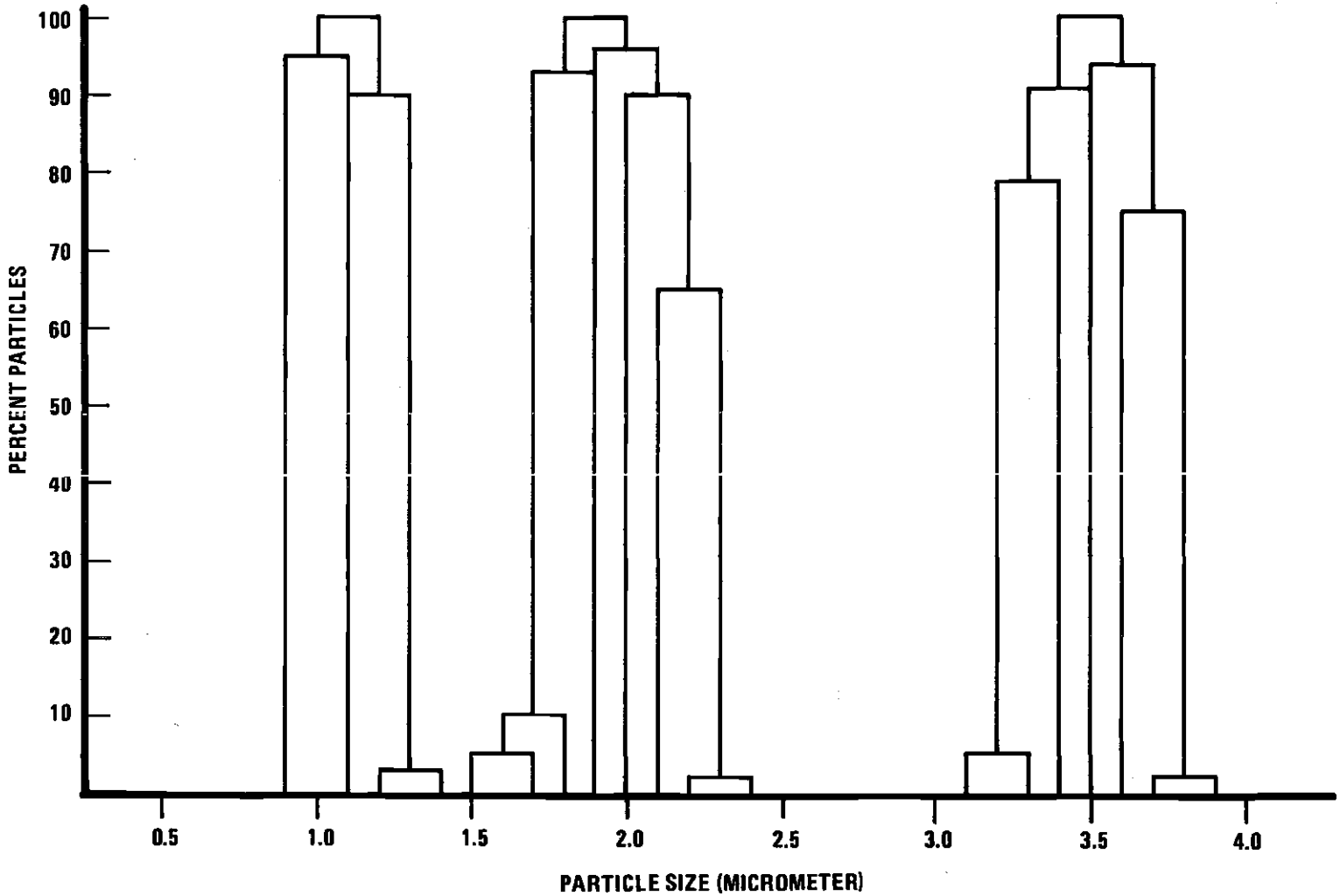

Figure 5. Size Distribution of Polyvinyltoluene Latex Microspheres as Determined by a Coulter Counter Model B with a 100 Micrometer Aperture.

Table 5. Results of Size Distribution Determinations With Polyvinyltoluene Latex Microspheres.

Size Range* (Micrometer)	Mean Diameter (Micrometer)	Background Count (per ml)	Total Count (per ml)	Percent Particles
1. For Latex Microspheres Labelled 1.011 ± 0.005 μ				
0.5-0.7	0.6	146523	149780	--
0.6-0.8	0.7	129514	133452	--
0.7-0.9	0.8	24415	37252	--
0.8-1.0	0.9	12754	32417	--
0.9-1.1	1.0	2010	25555	95
1.0-1.2	1.1	1721	27090	100
1.1-1.3	1.2	1751	24126	90
1.2-1.4	1.3	1594	2406	3
2. For Latex Microspheres Labelled 2.051 ± 0.018 μ				
1.5-1.7	1.6	477	2280	5
1.6-1.8	1.7	105	3756	10
1.7-1.9	1.8	---	35021	93
1.8-2.0	1.9	---	37565	100
1.9-2.1	2.0	---	36124	96
2.0-2.2	2.1	---	33915	90
2.1-2.3	2.2	---	24485	65
2.2-2.4	2.3	---	851	2
3. For Latex Microspheres Labelled 3.49 ± 0.017 μ				
3.1-3.3	3.2	59	1220	5
3.2-3.4	3.3	---	19150	79
3.3-3.5	3.4	---	22056	91
3.4-3.6	3.5	---	24205	100
3.5-3.7	3.6	---	22840	94
3.6-3.8	3.7	---	18291	75
3.7-3.9	3.8	---	484	2

*See Table 4 for instrument settings.

percent of the spores stained only faintly green and began to take up methylene blue stain around the edges as seen through the oil immersion lens. An occasional vegetative cell was also observed at this time. During a 10 minute interval between 13 and 13.5 hours there was a total loss of stainability with malachite green and 80 to 90 percent of the spores stained with methylene blue. Emergence and elongation of the vegetative cells followed and approximately 14.5 hours after inoculation, the first division occurred. About 10 percent of the cells were observed to divide before this time. The degree of synchrony was estimated by calculating a synchronization index (SI) according to Scherbaum (1964):

$$SI = 1 - \frac{t + g(2-n)}{1.12\ g} \qquad (1)$$

where t is the time in minutes during which synchronous or synchronized division occurs; g, the generation time in minutes of an asynchronous culture in its exponential phase of growth (designated gt by Scherbaum), and

$$n = 1 + \frac{\text{number of cells in synchrony}}{\text{total number of cells}} \qquad (2)$$

Points A through E in Figure 6, 7, and 10 are inflection points which were determined by drawing a tangent to the growth curves parallel to a straight line with a slope of the average generation time. The time intervals on the abscissa between points A and B, and C and D represent t in Eq. (1). The number of cells in synchrony in Eq. (2) were computed by direct readings from the ordinate by taking the difference in the cell numbers at the inflection points A and B, and C and D. The total number

of cells in Eq. (2) is represented by the number of cells from one generation to the next. The average generation time (g) of Clostridium botulinum, type F, strain Langeland growing in TSB^+ at 30 C was found to be 80 minutes.

As seen in Figure 6, outgrowth of the spores was partially synchronized with an estimated SI = 0.55 for the first synchronous step. Synchrony deteriorated rapidly to an estimated SI = 0.30 for the second step. Total counts and viable counts were in close agreement until late in the decelerating log phase of growth when autolysis commenced. The viable count reached a maximum of about 4.5×10^7 cells per ml in the stationary phase and remained at that level for approximately 90 minutes after which the count declined gradually. The total count reached a maximum of about 5×10^7 particles per ml and remained there for several hours before a very slow decline commenced. The optical density followed a pattern similar to that of the total count once the stationary phase of growth was reached.

Transfer of the partially synchronous culture into fresh TSB^+ after the first doubling in the number of cells resulted in an improved synchrony (Figure 7). After a lag period of about 30 minutes the first synchronous step occurred with a SI = 0.69 and a second step with a SI = 0.52. A third step was only indicated. Total and viable counts were in close agreement during the first synchronous step. Differences, however, began to be noticeable during the second step, the total count of particles becoming larger than the viable count. Information for calculating the degree of synchrony is summarized in Table 6.

The Stationary Phase Method

The stationary phase method did not result in synchronous growth

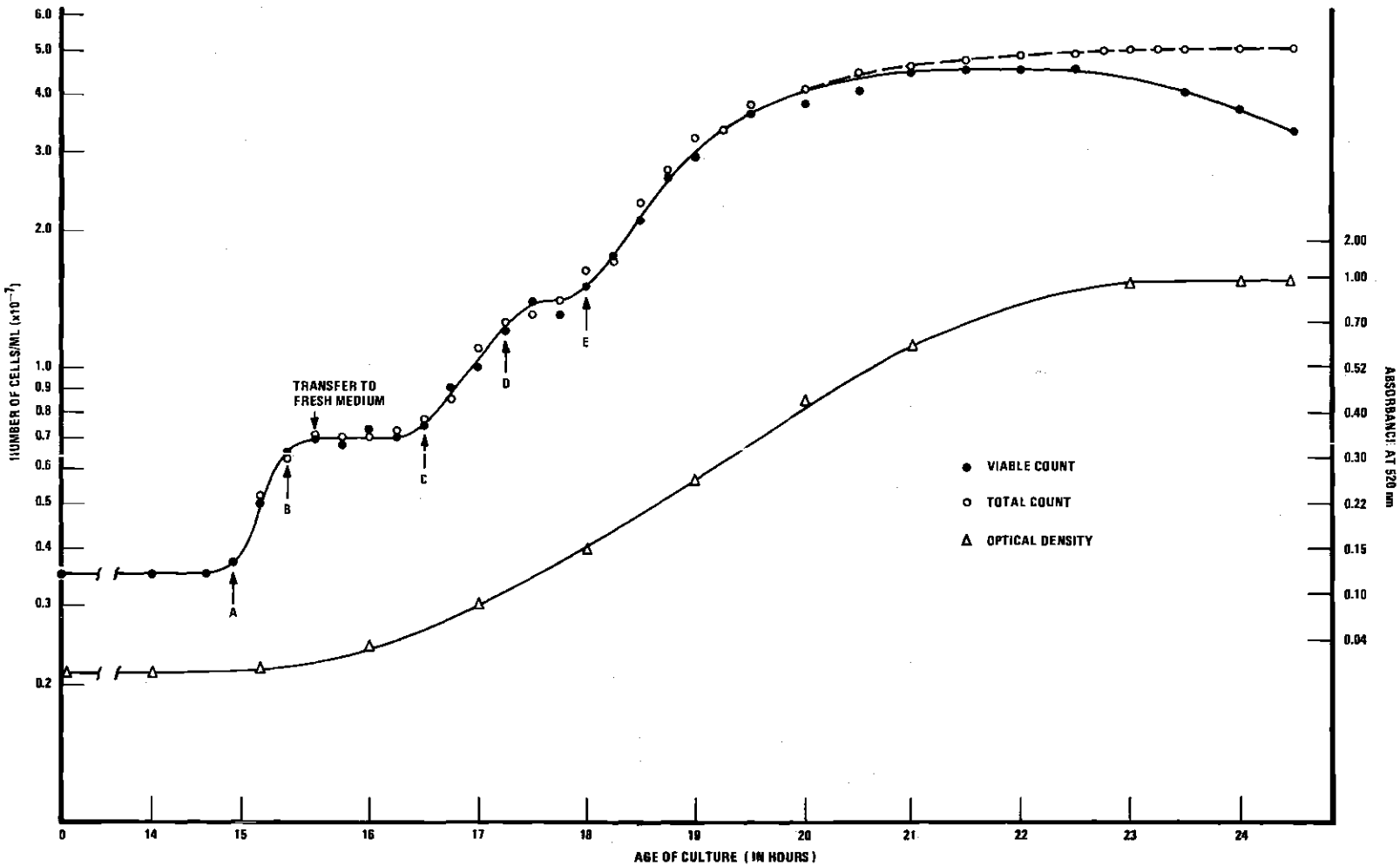

Figure 6. Partially Synchronous Growth of Vegetative Cells of Clostridium botulinum, Type F, Strain Langeland Following Outgrowth of a Spore Inoculum in TSB^{+} at 30 C.

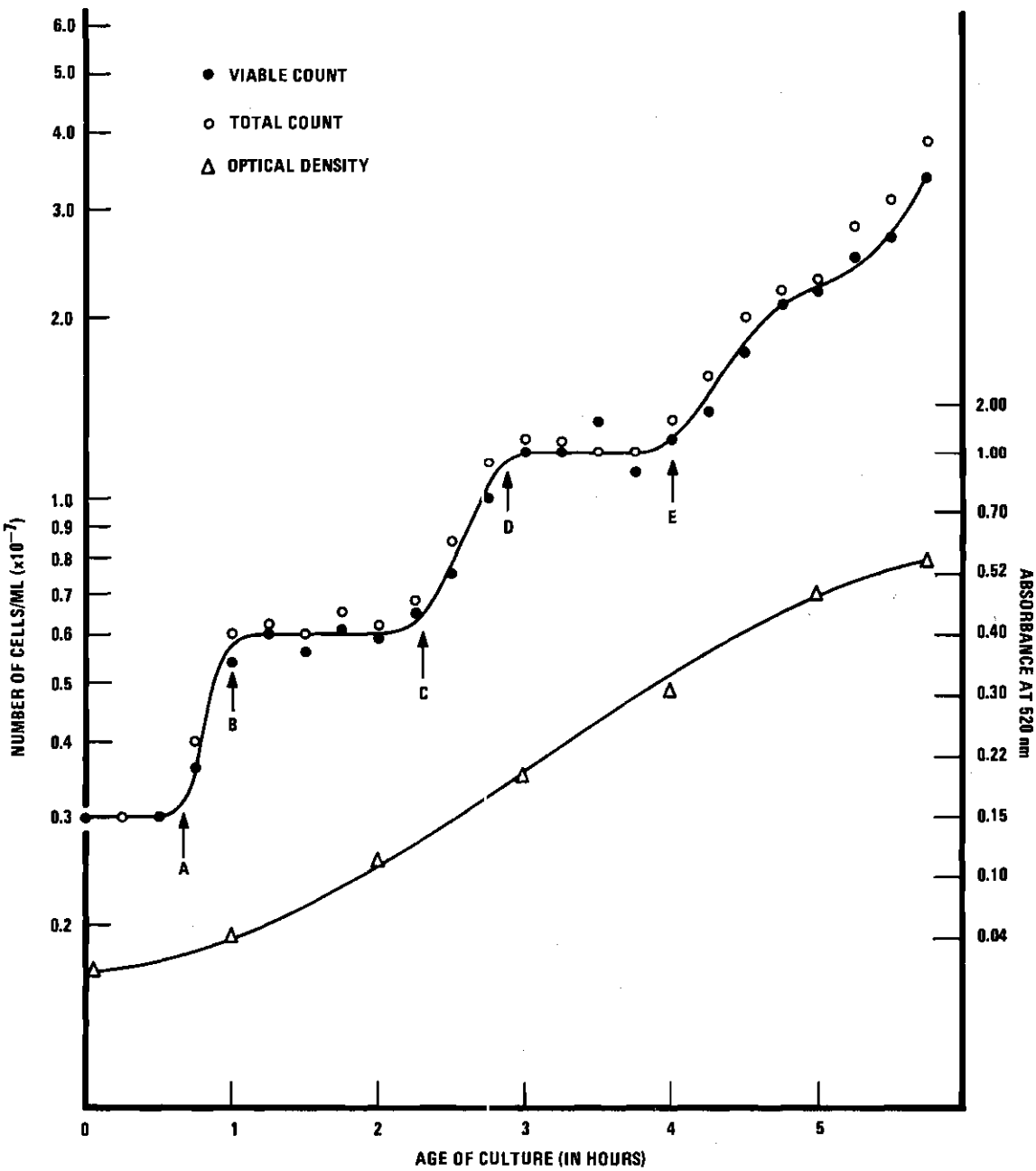

Figure 7. Improvement in Growth Synchrony of a Culture of Clostridium botulinum, Type F, Strain Langeland as a Result of Transferring a Partially Synchronous Culture into Fresh Medium (see Figure 6).

Table 6. Calculation of the Synchronization Index (SI)* of Clostridium botulinum, Type F, Strain Langeland.

Reference	Inflection Points	t (Minutes)	n	SI
Figure 6	A → B	30	1.86	0.55
	C → D	45	1.73	0.30
Figure 7	A → B	20	1.90	0.69
	C → D	30	1.83	0.52
Figure 10	A → B	35	1.85	0.48
	C → D	55	1.80	0.21

*According to Scherbaum (1964).

of Clostridium botulinum, type F, strain Langeland. A culture transferred repeatedly from the beginning of the stationary phase of growth (see Figure 3, point A) into fresh TSB^{+} showed a typical sigmoidal growth response as demonstrated in Figure 8. Viable counts indicated a short lag period of approximately 30 minutes, followed by exponential growth with an average generation time of 80 minutes. A maximum viable count of about 4.5×10^7 cells per ml was attained in the stationary phase. The total count was noticeably higher than the viable count during lag and early log phase. However, the counts approached each other during most of the remaining exponential and early decelerating log phase of growth. Then both counts began to separate again with the total count reaching a maximum of 5×10^7 particles per ml in the stationary phase.

Subcultures taken from points B through F (see Figure 3), each separated from the next by 25 minutes, differed from culture "A_3" of Figure 8 only in two respects: (i) increasingly longer lag times, and (ii) increasingly larger differences between total and viable counts except for the maxima reached in the stationary phase of growth. These maximum counts remained at $4.0 - 4.5 \times 10^7$ viable cells per ml and 5×10^7 total particles per ml for all cultures. Subcultures taken from points G (mid-decelerating log phase) and H (end of exponential phase) differed from culture "A_3" of Figure 8 only in that they showed no discernible lag phase.

Cold Shocking

Cultures which were cold shocked at 4 C differed in their growth responses depending on the length of time of the shock and the phase of growth from which they were taken. Figure 9 shows the effect of cold

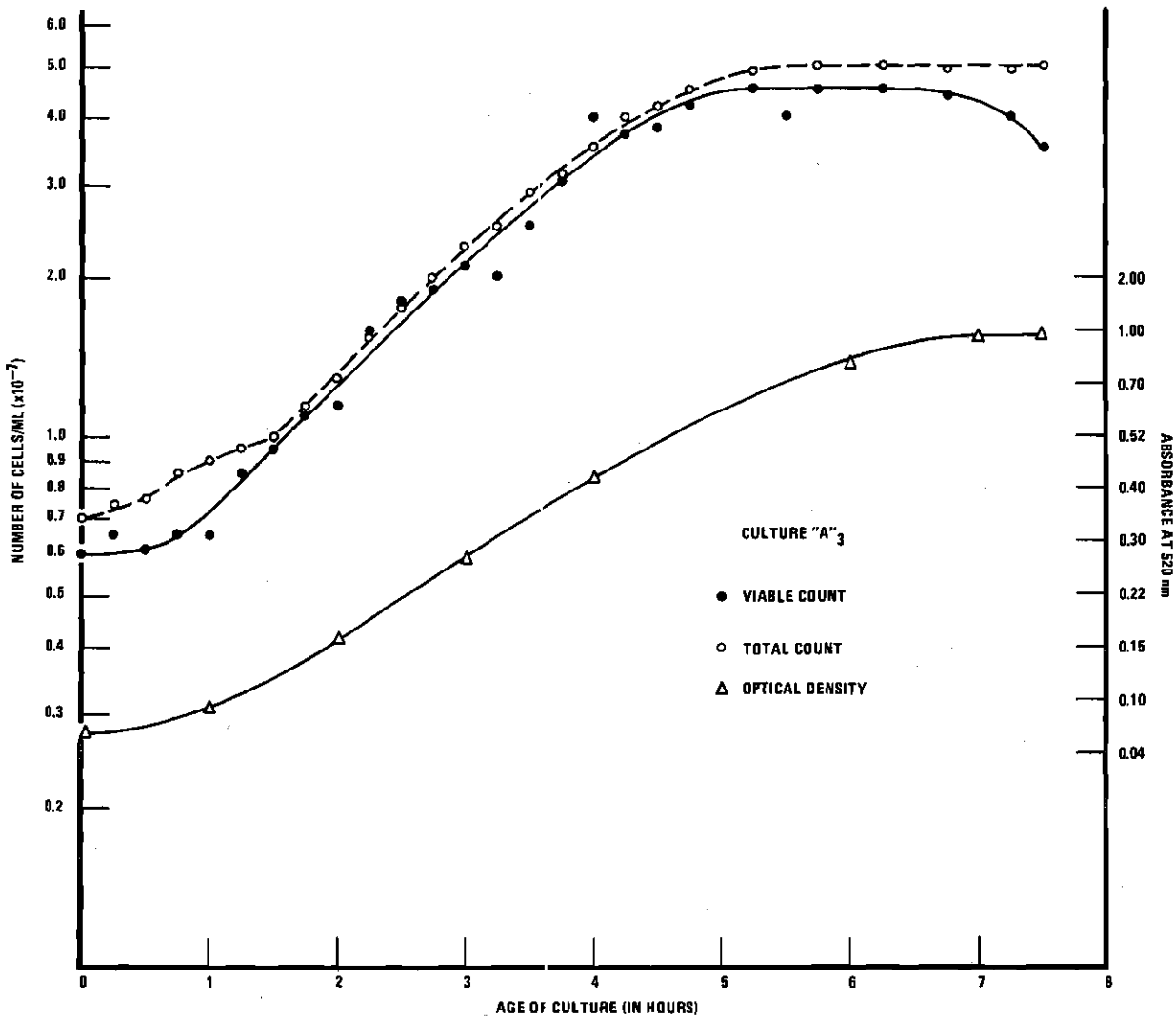

Figure 8. Typical Growth of Clostridium botulinum, Type F, Strain Langeland seen When the "Stationary Phase Method" (see point A in Figure 3) was Used to Achieve Synchrony.

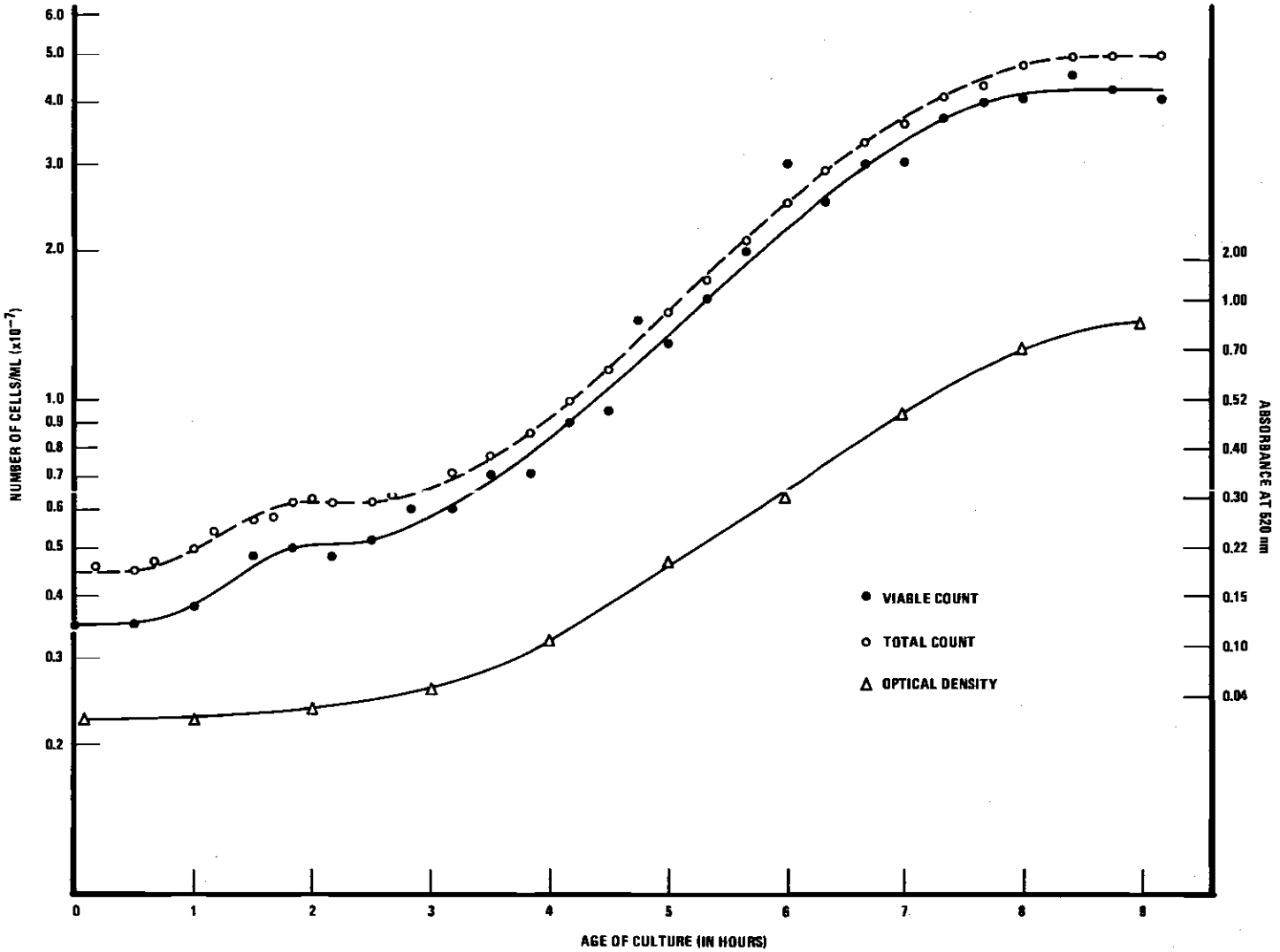

Figure 9. Effect of Cold Shocking a 30 C Stationary Phase Culture of Clostridium botulinum, Type F, Strain Langeland at 4 C for 60 Minutes Followed by Reinoculation into Fresh TSB^{+} at 30 C.

shocking a stationary phase culture (one hour into stationary phase as shown in Figure 4) for 60 minutes at 4 C. This length of time of cold shocking produced the most pronounced step towards synchronized growth; 15 minutes of cold shocking resulted in a growth response similar to that seen in Figure 8 with the exception of a much longer phase of adjustment (lag phase plus acceleration phase), lasting from 120 to 150 minutes, before reaching exponential growth; 30 minutes of cold shocking produced the first indication of a step towards synchrony which was amplified at 45 minutes and finally resulted in the most distinct step at 60 minutes as demonstrated in Figure 9. Further cold shocking reversed the synchronizing effect. At 100 minutes the step began to flatten out and at 120 minutes of cold shocking it had disappeared altogether leaving a very long phase of adjustment of approximately 240 minutes in its place. The phase of adjustment increased to 360 to 390 minutes when cultures were cold shocked for 180 minutes. Stained smears of cell samples showed no visible signs of ill effects from cold shocking for periods of 15 to 30 minutes. At 45 to 100 minutes, however, a few unusually long cells without division septa were observed. These cells disappeared during exponential growth. At 120 to 180 minutes of cold shocking the number of long, non-dividing cells increased significantly. These did not disappear during steady state growth. Many cells also appeared curved or with diffuse and pointed ends. A reproducible effect of cold shocking for 180 minutes was the occurrence of forespore-like swelling of 50 to 70 percent of the cells during the first two hours after restoration to the 30 C incubation temperature. However, maturation into refractile spores did not follow.

Figure 10 shows the effect of cold shocking an exponentially growing culture (end of exponential phase) for 45 minutes at 4 C. After a lag of about 40 minutes, synchronized growth ensued with an estimated SI = 0.48 for the first step and SI = 0.21 for the second one. Information for calculating the degree of synchronization is summarized in Table 6. Total and viable counts were in somewhat closer agreement than for the cold shocked cultures taken from the stationary phase (compare Figure 9 with Figure 10). Cold shocking an exponentially growing culture for 15 and 30 minutes brought about a growth response similar to that seen in Figure 8 with a phase of adjustment lasting from approximately 50 to 80 minutes. As far as the growth curve was concerned, cold shocking an exponentially growing culture for 60, 120, and 180 minutes affected only the length of the phase of adjustment increasing it from about 120 to 150 to 210 minutes, respectively. Stained smears showed no visibly irregular morphology until 60 minutes of cold shocking were used. At this time long, non-septated cells were observed whose frequency of occurrence and persistence throughout the growth cycle increased with longer periods of cold shocking. Also, curved and very short cells appeared in addition to those with diffuse and pointed ends. The formation of forespore-like swelling was not observed as in the case of cold shocking stationary phase cultures.

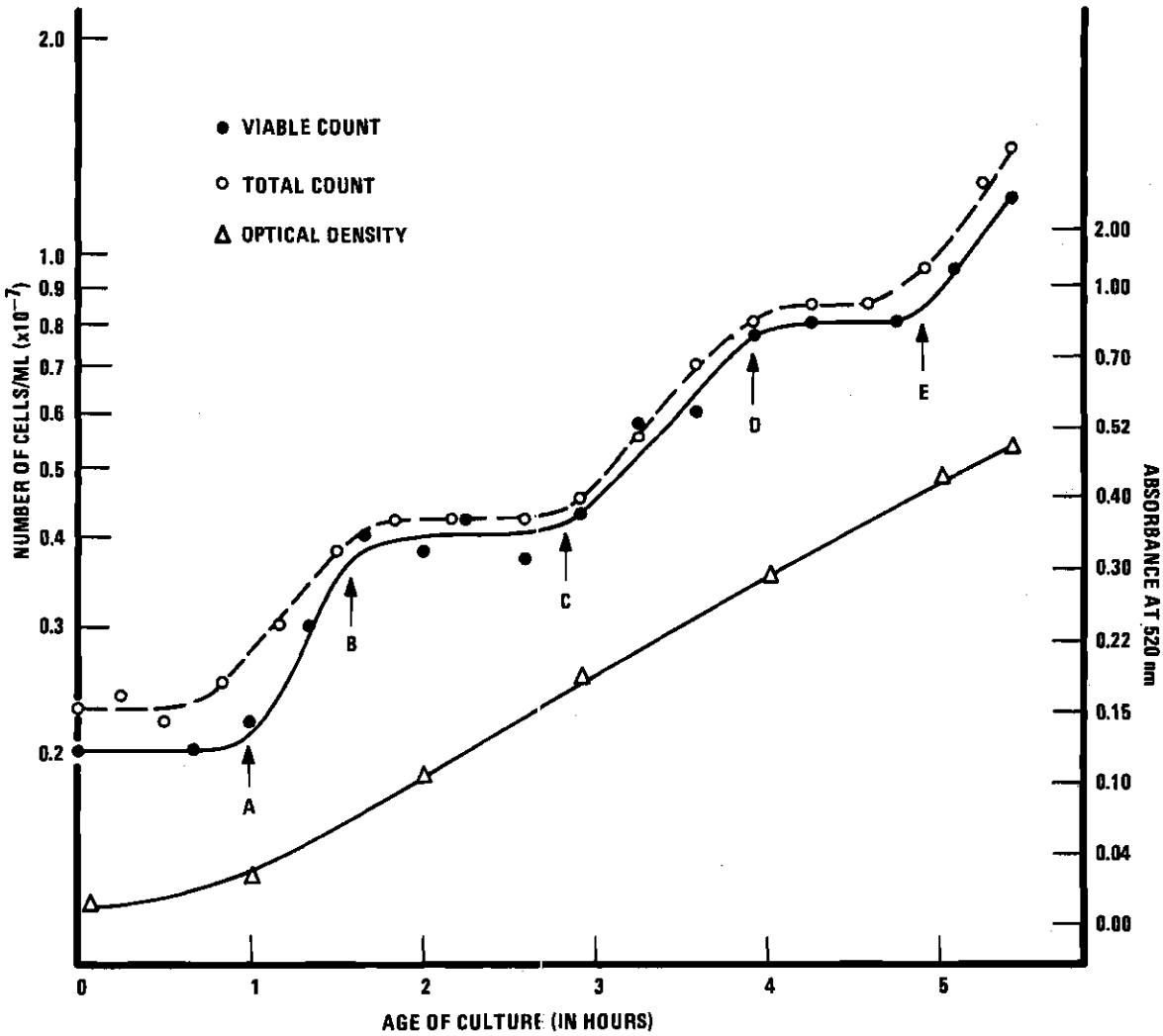

Figure 10. Effect of Cold Shocking a 30 C Exponential Phase Culture of Clostridium botulinum, Type F, Strain Langeland at 4 C for 45 Minutes Followed by Reinoculation into Fresh TSB^{+} at 30 C.

CHAPTER IV

DISCUSSION

Equipment

The use of automated and semi-automated equipment proved to be essential for the execution of the synchronous growth experiments. The nature of the investigation required large numbers of experiments in order to find the proper cultural conditions which would produce synchrony and to establish the reproducibility of a method.

The growth of a bacterial culture in a large vessel is commonly followed by removing small samples at specific intervals of time for the determination of total and viable cell counts as well as optical density. In synchronous growth experiments these intervals of time have to be closely spaced in order to detect the stepwise increase in the number of cells. If the total counts are made with a Petroff-Hausser counting chamber, a very tedious procedure, it usually becomes necessary to store the samples (properly diluted with formaldehyde to arrest growth) at refrigeration temperature until counts can be made. Also, the results of viable counts in solid media are not available until several days after the completion of an experiment because of the length of incubation time necessary for the formation of countable colonies. Therefore, the only immediate means of determining the progress of a growing bacterial culture is the measurement of optical density. It must be noted that this method gives only an indirect indication of growth since it measures the increase

in bacterial mass which is always linear during steady state growth. A stepwise increase, by definition synchronous or synchronized growth, is therefore not detectable by measurements of optical density.

When I applied the conventional approach of monitoring growing cultures of bacteria to the synchronization studies of Clostridium botulinum, type F, strain Langeland, I soon discovered that this approach was too time consuming and, in part, unreliable since I was unable to perfect a technique giving reproducible results using the Petroff-Hausser bacteria counter. The incorporation of a Technicon Auto-Analyzer and a Coulter Counter into the experimental procedure reduced the time required for each experiment considerably and made information on total cell counts and on optical densities immediately available. Nevertheless, sampling for total and viable counts was not feasible at intervals of less than 15 minutes. The time could be shortened to 10 minutes if the viable cell count was omitted. Until a method was established as reproducible this omission was found to be practical, since the preparation of fresh pork infusion medium for viable counts is a lengthy and expensive procedure.

Coulter Counter Model B

The instrument determines the number and size of particles suspended in an electrically conductive substrate. The suspension of particles is drawn through a small aperture with an immersed electrode located on either side of it. A particle passing through the orifice displaces a volume of electrolyte equivalent to its own volume, momentarily changing the resistance between the electrodes. The change in resistance produces a voltage pulse of a magnitude proportional to the volume of electrolyte displaced by the particle. The pulse is amplified and displayed on an

oscilloscope. Dual threshold circuits with adjustable voltage levels allow an operator to set lower and upper thresholds so that only pulses falling between these levels are counted. This corresponds to counting an equivalent range of particle volumes which may then be translated into an equivalent range of spherical diameters.

Sources for counting errors are numerous but may be corrected or avoided. Errors may be due to primary and secondary coincidence, relaxation times of the instrument's circuitry, background noise from the circuitry, and outside electrical interference. Primary coincidence is defined as the simultaneous passage of more than one particle through the aperture. It may be avoided by proper dilution of the particle concentration in the counting medium (approximately 5 to 20,000 particles per ml). Keeping the concentration of particles at a low level will also correct counting errors due to the electrical and mechanical limitations of the instrument's circuitry (relaxation time). Secondary coincidence is a result of the time required for a voltage pulse to build up and relax again; the time increases as the size of the particles increases. This source of error is probably negligible for bacteria; for larger particles, the counting error may again be eliminated by dilution. The influence of outside electrical interference on total count determinations is most difficult to avoid even with proper grounding and sophisticated shielding methods such as placing the entire instrument into a Faraday's cage. Interferences can be detected by listening to changes in the normally constant count rate (which may slow down or speed up and, in cases of severe interference, stop altogether) and by observing loss of the pulse pattern or sections of it on the oscilloscope. I found total

count determinations during normal working hours frustrating and frequently impossible. Counts in one 0.5 ml sample were not reproducible in the next 0.5 ml, and large sections of the pulse pattern were often blocked out, usually in rapid rhythmic intervals. As a consequence, most of my experiments were done during the (electrically) quiet night hours and weekends.

Kubitschek (1958) was the first to show that the Coulter Counter instrument, originally designed as a diagnostic tool for the rapid counting of blood cells, could be adapted to the much lower size range of bacteria. He used a custom-made 10 micrometer aperture. Other investigators (Helmstetter and Cummings, 1963; Allison et al., 1962; Swanton et al., 1962; Toennies et al., 1961; Lark and Lark, 1960) have since applied the Coulter Counter to investigations of bacterial growth kinetics and total cell counts using a 30 micrometer orifice which, to my knowledge, is the smallest commercially available aperture. For this study, only a 100 micrometer aperture was at my disposal. It was essential to determine whether particles as small as bacteria could be counted reliably using such a large orifice since the literature gave no reference to that effect. In the case of the Model A Coulter Counter, experience has shown (personal communication, J. H. Burson and E. Y. H. Keng, Department of Chemical Engineering, Georgia Institute of Technology) that particles of a size less than two percent of the aperture size can no longer be counted because of interference from thermal effects (boiling). Boiling in the aperture is made visible by the appearance of so called "streaming," a flow of tiny gas bubbles emanating from the aperture. This effect was not observed with the Model B Coulter Counter even for instrument settings

designed to count particles of less than one micrometer in diameter. However, these latter particles could not be counted due to the interference of the electrical background noise. This noise appeared on the oscilloscope as a horizontal band approximately 1 to 2 millimeters in thickness. A freshly filtered sample of electrolyte showed only occasional pulses above that line; a sample of vegetative cells of Clostridium botulinum, type F, strain Langeland exhibited a solid pulse pattern. When the lower and upper thresholds were set for counting a certain size range of cells the pulse pattern between the threshold levels illuminated, thereby creating a so called "window." This window moved up and down in the pulse pattern as the size of the cells to be counted increased or decreased. The lower threshold setting of the lowest size range of particles counted in this study (1.0 to 1.5 micrometers with a mean diameter of 1.25 micrometers) was distinctly separated from the base line of the electrical background noise. However, when particles of less than one micrometer in size were counted the lower threshold level began to merge with the background noise and particle count and noise count became indistinguishable from each other.

In the absence of electrical interference, I found total count determinations with the 100 micrometer aperture precise and highly reproducible. As far as accuracy is concerned, it should be pointed out that the total counts shown in Figures 6, 7, 8, 9, and 10 may be too low by as much as 3 percent during the initial phases of growth and approximately 20 percent in the decelerating log and stationary phases because no corrections were made for coincident passage of cells.

Technicon Auto-Analyzer

The instrument assembly is a most versatile tool for automated chemical analyses. Its basic components are a proportioning pump, colorimeter, and recorder. Extensive use of this instrumentation for the automated determination of bacterial growth curves (by measuring optical density vs time) is not yet realized; the Technicon Auto-Analyzer bibliography 1957/1967 lists only one reference (Anonymous, 1965).

I found the instruments easily assembled into a functional unit which required very little maintenance and performed in excess of 200 hours before the special tolerance tubing across the platen of the proportioning pump showed signs of weakening and stretching and needed replacing. All other tubing (standard transmission tubing, 0.065" I.D.) did not require replacing. Sterilization was at first accomplished by flushing the tubing with sterile deionized water shortly before and after an experiment. Experience soon showed, however, that water alone did not sufficiently clean the inside of the tubing; cells and cellular debris became attached to the inside walls during growth experiments, in particular during the late log and early decelerating log phases. Debris accumulated in the horizontal arm of the C-5 debubbler located at the inlet to the flowcell of the colorimeter (see Figure 1) causing a gradual restriction of the culture flow. Occasionally the debris broke loose and was swept back into the culture vessel. The resulting growth curve was "saw-tooth like" in shape as demonstrated in Figure 11. The obstacle was overcome by first flushing the tubing with an acid cleaning solution* and then thoroughly rinsing with sterile deionized water after each experiment.

*Hartman-Leddar Co., Philadelphia, Pennsylvania.

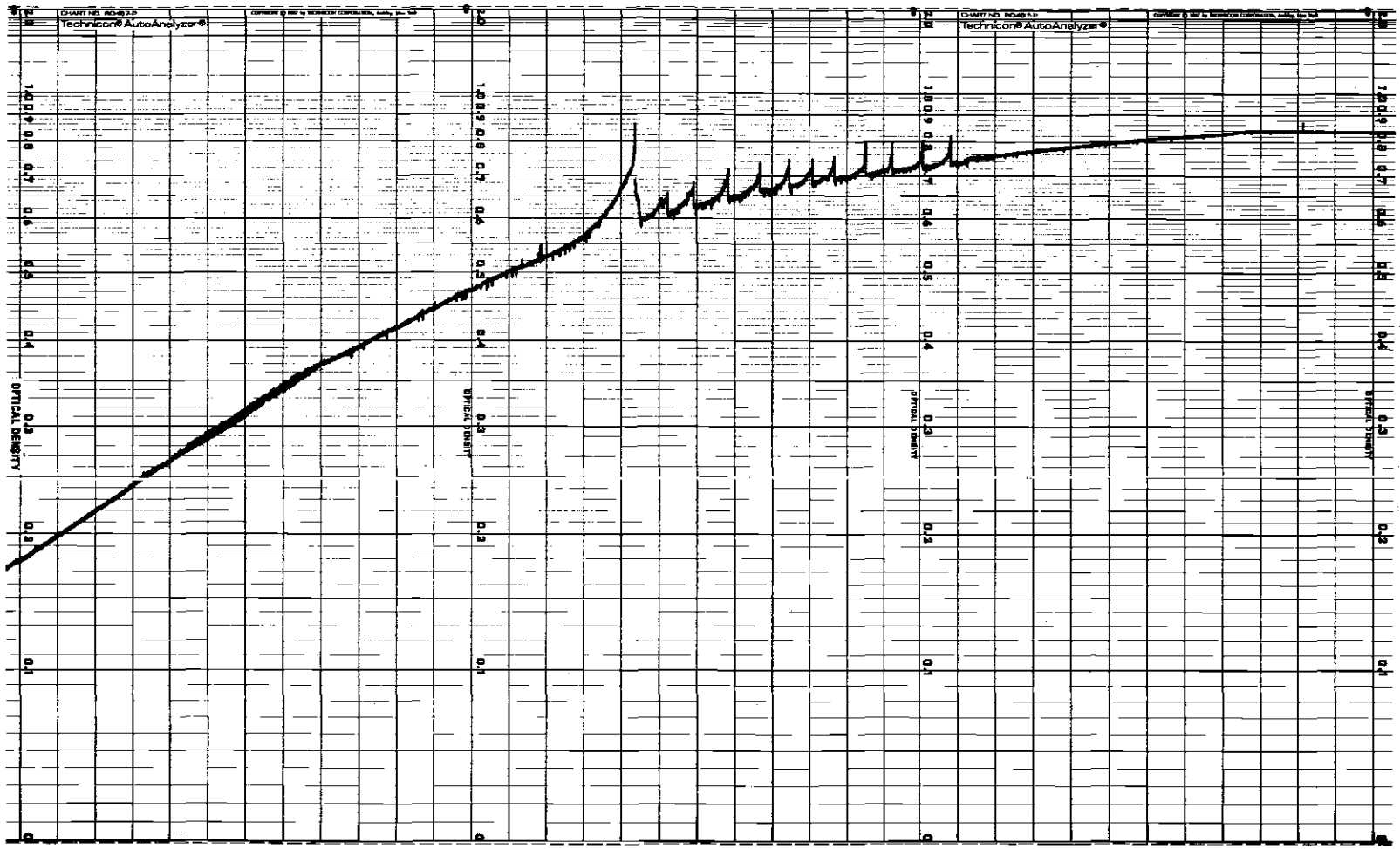

Figure 11. Continuous Optical Density Record of a Growing Culture of Clostridium botulinum, Type F, Strain Langeland Using an Auto-Analyzer Assembly. The Interruptions in the Growth Curve Result from the Accumulation and Sudden Breaking Away of Debris in the Debubbler Installed at the Inlet to the Flowcell of the Technicon Colorimeter (see Figure 1).

Sterile deionized water was again used just prior to the next experiment.

A second difficulty in obtaining a smooth growth curve arose from the formation of gas which first appeared in very small quantities during the mid-exponential phase and reached maximum production when a growing culture was about to enter the stationary phase. An example of the resulting growth curve is shown in Figure 12. In order to remedy the disturbing effect of gas production on the continuous recording of a growth curve, a C-5 debubbler was installed at the inlet to the colorimeter (see Figure 1). The debubbler was able to divert the small amounts of gas produced during and immediately following steady state growth to a waste flask (see Figure 1 or Figure 2), but relatively large quantities of gas were still able to flow through the colorimeter during the vigorous fermentation of yeast extract and dextrose in the decelerating log phase of growth so that the resulting growth curve showed numerous spikes (Figure 13). The installation of a second debubbler (see modified C-5 debubbler in flow diagram of Figure 1) finally accomplished the recording of smooth growth curves of Clostridium botulinum, type F, strain Langeland (Figure 14). This second debubbler was made by simply straightening that arm of a regular C-5 debubbler which is normally connected to the waste tubing (arm C, "General Operating Instruction Manual," Section C-R, p. 4). Figure 14 demonstrates another significant point: the growth curve afforded an immediate check on the state and reproducibility of cultural conditions. Excepting the phase of adjustment, the curve was virtually identical for most experiments undertaken in this study. During the early phase of the investigation rigorous control over anaerobiosis was lacking and the resulting growth curves varied considerably in their slopes (compare

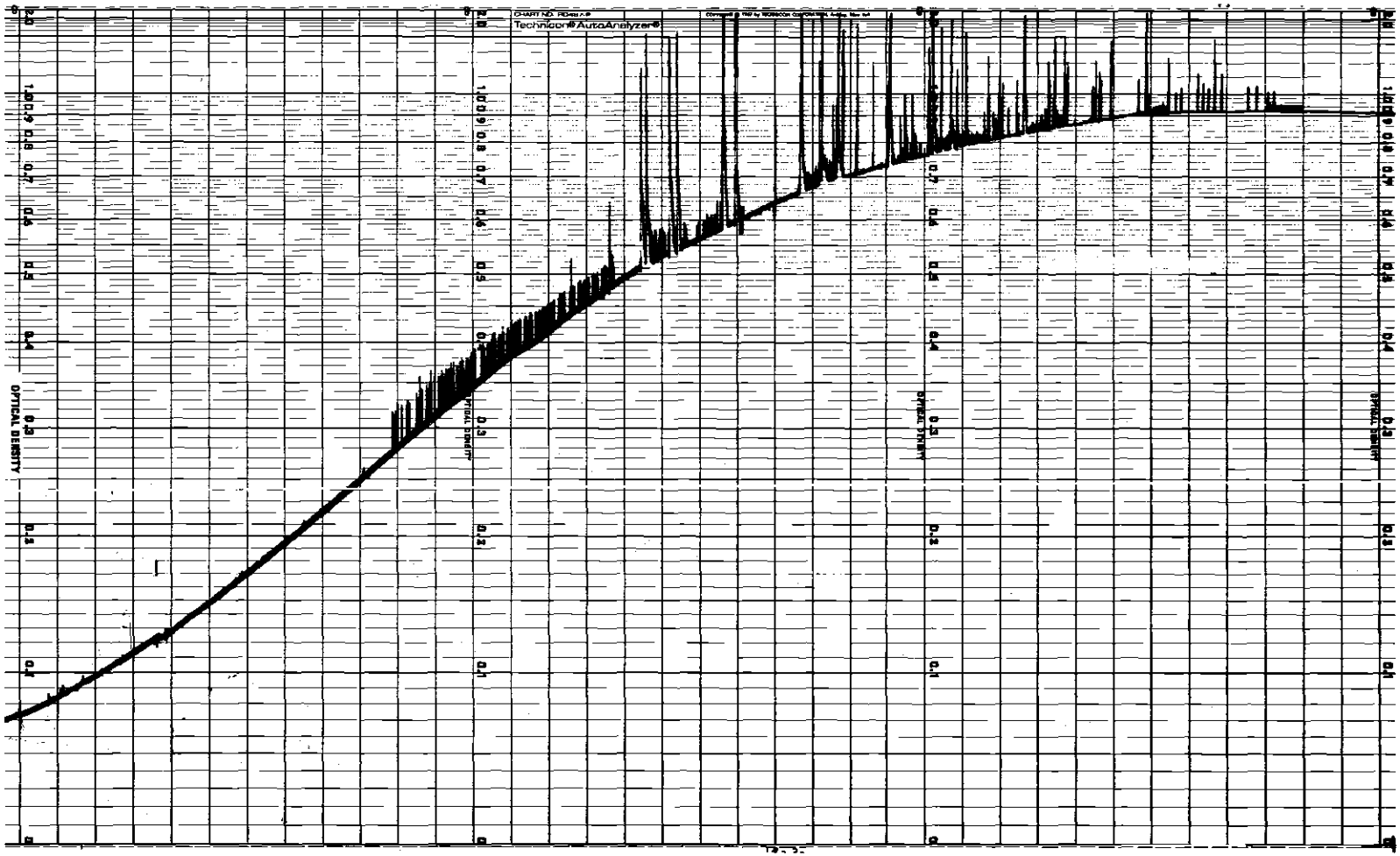

Figure 12. Continuous Optical Density Record of a Growing Culture of Clostridium botulinum, Type F, Strain Langeland Using an Auto-Analyzer Assembly Without Debubblers.

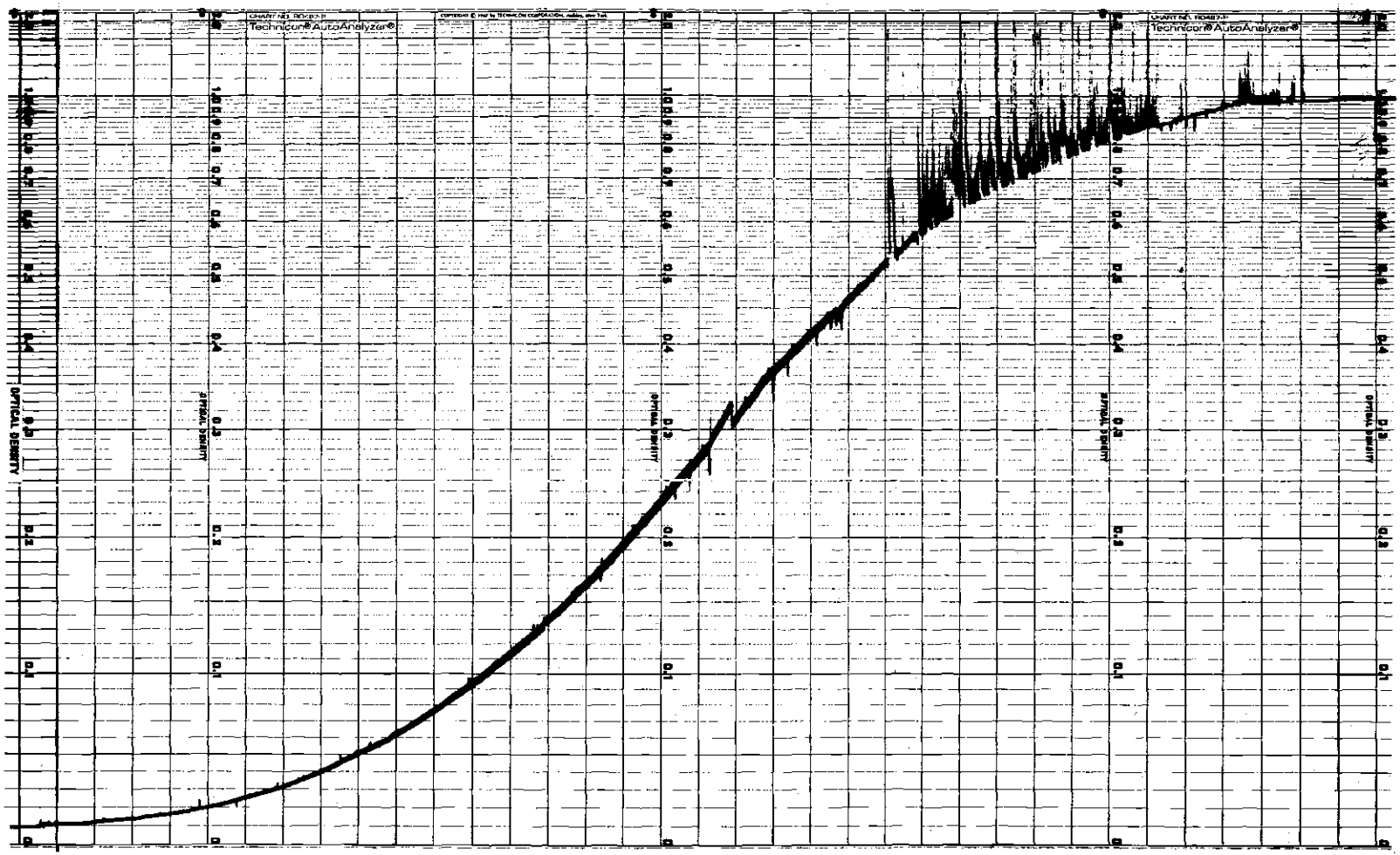

Figure 13. Continuous Optical Density Record of a Growing Culture of Clostridium botulinum, Type F, Strain Langeland Using an Auto-Analyzer Assembly With One Debubbler.

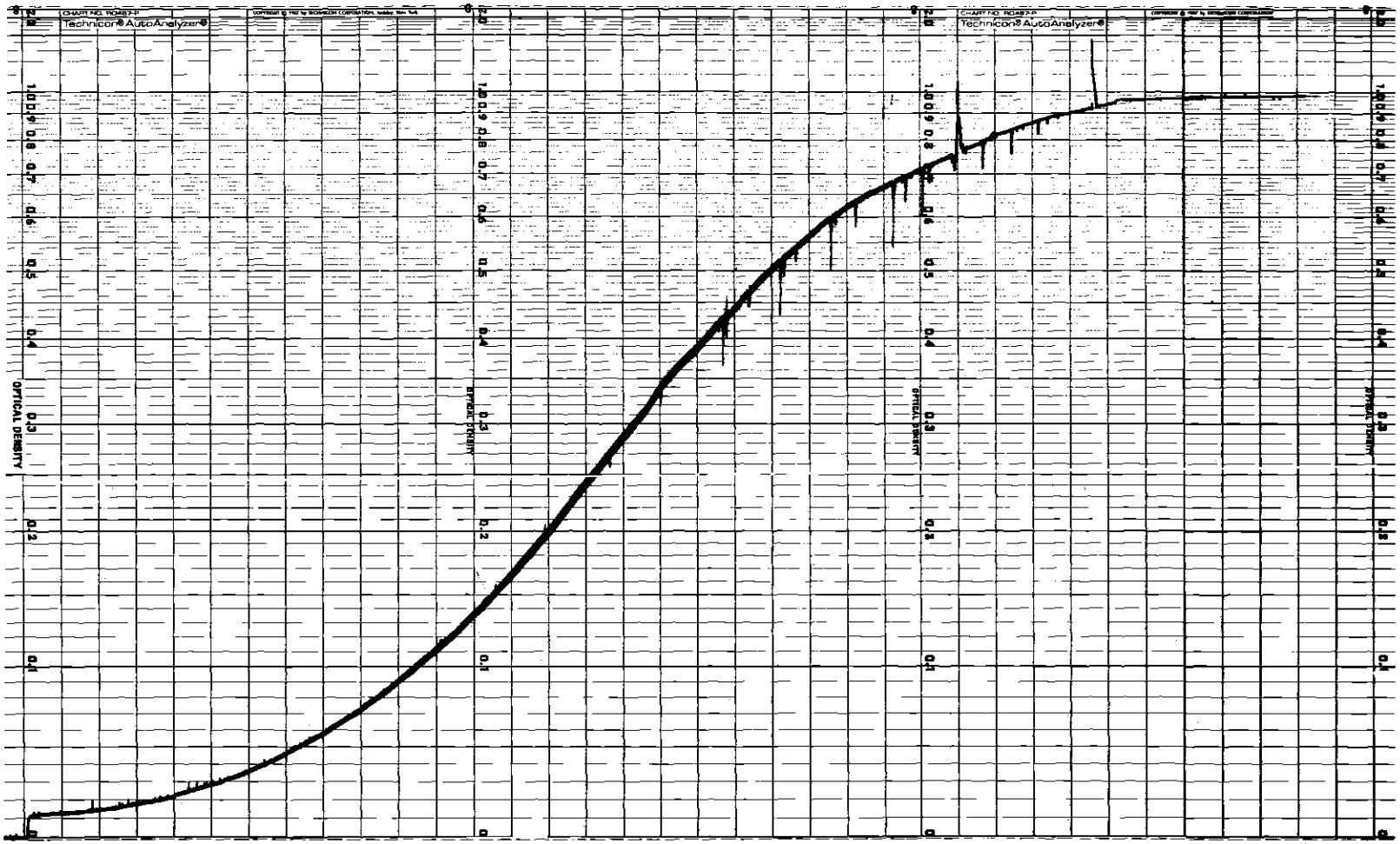

Figure 14. Continuous Optical Density Record of a Growing Culture of Clostridium botulinum, Type F, Strain Langeland Using an Auto-Analyzer Assembly With Two Debubblers.

Figure 11 with Figure 14). At first, TSB+ was used as the growth medium without the addition of sodium thioglycollate (an oxygen scavenger), resulting in curves with shallow slopes and maximum absorbance of 0.6 in the stationary phase. Apparently, the continuous stirring of a growing culture permitted oxygen to dissolve into the medium. The addition of 0.1 percent sodium thioglycollate to the growth medium improved anaerobiosis with the result that growth curves with steeper slopes were obtained. Consistent difficulties were encountered with this chemical obtained from Fisher Scientific Company or Baltimore Biological Laboratories (BBL). The chemical either disintegrated during autoclaving and lost its function as a reducing agent or, when added as a filter sterile solution, was toxic to the test organism. Less difficulties were encountered with sodium thioglycollate marketed by Difco Laboratories, although occasional lots were found to be unstable during autoclaving, none were toxic when filter sterile solutions were added to the growth medium. The method which was reproducible and finally adopted combined the addition of filter sterile sodium thioglycollate with continuous bubbling of nitrogen.

Since Clostridium botulinum, type F, strain Langeland is a pathogenic organism, safety was a major concern. It has been my experience that, if the tubing is assembled and fittings and connections are made according to the instruction manual, leaks of any type are very unlikely to occur. Nevertheless, proportioning pump and colorimeter were placed on a metal tray as a precautionary measure. The small space between the water bath and the proportioning pump was bridged with aluminum foil slanted from the top of the water bath down to the tray (see Figure 2),

so that any accidental leakage from the connections between culture vessel and pump tubing was diverted into the tray for autoclaving. The weakest point in the system, with respect to safety, was found to be the proportioning pump. It is essential that the two metal strips on either side of the platen are in perfect condition so that the rollers can press down on the tubing at right angles. If one of the metal strips is weakened it may begin to bulge, causing the pump tubing to stretch unduly to one side or the other and finally to tear. As a means of protection against such an eventuality, the pump was enclosed in a casing made of clear plastic (aluminum foil or a plastic bag will serve the same purpose).

Synchronous and Synchronized Growth Experiments

Initiation from Spore Inoculum

The initial experiments to synchronize both vegetative growth and sporulation of Clostridium botulinum, type F, strain Langeland were unsuccessful attempts to duplicate existing methods for Clostridium roseum (Halvorson, 1957) and Clostridium botulinum, 62-A (Day and Costilow, 1964). The next step consisted of exploring the possibility of germination synchrony. Since the literature gave no reference on the subject as far as Clostridium botulinum was concerned, I had to rely on previous experience with the type F organism. First, I tried trypticase soy broth as the germination and growth medium, but found the time of outgrowth lasting as much as 3 hours from the appearance of the first vegetative cells until the disappearance of the last spores (15 to 18 hours after inoculation). However, I discovered that: (i) heating the spores for 15 minutes at 85 C prior to inoculation did not affect the

germination time although heat shocking is a well known method of inducing rapid germination in aerobic sporeformers (I continued the practice as a means of destroying vegetative cells including those of possible contaminants), (ii) spores of Clostridium botulinum, type F, strain Langeland will not germinate in the presence of dissolved oxygen in the medium, and (iii) outgrowth is delayed considerably by continuous stirring of the spore inoculum even under strictly anaerobic conditions.

Next, I attempted to reduce the lag time between the appearance of the first vegetative cells and the disappearance of the last spores by adding yeast extract, known to stimulate germination, and sodium thioglycollate. The attempt resulted in partial germination synchrony. Unfortunately, the degree of synchrony could not be improved. The reason may lie in the fact that the spore crop had been produced in a batch culture. Batch cultures are known to produce spore populations which are heterogeneous with regard to density (Tamir and Gilvarg, 1966; Church and Halvorson, 1959). Light spores have been found to differ in their germination properties from those of heavy spores (Tamir and Gilvarg, 1966). It was possible, however, to improve the degree of synchrony of the vegetative growth that followed the partial germination synchrony by diluting the cells into fresh medium after the first doubling in numbers had occurred (see Figure 7). The mechanism by which this dilution method improves synchrony is not known. The limited degree of improvement may perhaps be due to the effects of a slight temperature "shock" and a brief exposure to oxygen while the cells were being centrifuged and resuspended in fresm medium.

Stationary Phase Method

Cutler and Evans (1966) theorize that this method may be widely applicable to many bacterial species since apparently it entails only the transfer of an asynchronous population of cells from a particular time in the stationary phase of growth into fresh medium at about a sevenfold dilution. The procedure may be repeated in order to improve the degree of synchrony. Cutler and Evans showed that for E. coli K-12 Hfr, K-12 F^-, and B/r, and P. vulgaris the optimum harvest time was one generation into the stationary phase of growth. The investigators hypothesized that the synchrony may be due to the relationship between amino acid synthesis and DNA transcription activity. As growth conditions become unfavorable towards the stationary phase the size of the amino acid pool decreases in minute steps, which triggers a sequence of events: a gradual excess of unactivated t-RNA begins to complex with RNA polymerase thus lowering the DNA transcription rate, m-RNA concentration, and finally the rate of protein synthesis. As a consequence, cells in the decelerating log phase are growing faster than those already in the stationary phase. It is conceivable then that at some specific time in the stationary phase of growth most cells may exist momentarily in the same physiological stage. The existence of such a stage is thought by Cutler and Evans to be the basis for the synchronous growth which follows transfer of the cell population into fresh medium. Their method seems to be unique in producing cultures which grow both in cellular division synchrony and chromosomal synchrony. Supporting evidence for chromosomal synchrony is derived from studies on the sequential replication of the Bacillus subtilis W23 chromosome during exponential and stationary phases of growth (Tevethia

and Mandel, 1967; Yoshikawa et al., 1964; Yoshikawa and Sueoka, 1963). These investigators found that the chromosome of B. subtilis is in a completed, nonreplicating form when the cells are in the stationary phase of growth. When transferred to fresh medium replication is initiated by most cells at the same time, the replication being started from the origin and proceeding to the terminus of each chromosome in a sequential manner.

In spite of concentrated efforts, I was unable to synchronize the growth of Clostridium botulinum, type F, strain Langeland by using the stationary phase method. It is conceivable that the time interval during which the conditions for the initiation of synchronous growth are favorable may be very short for the type F organism and was simply missed. It is also possible that the technique of transferring the stationary phase cultures into fresh medium may have produced temperature and oxygen shocks of sufficient degrees to disturb the synchronizing mechanisms without affecting the growth response of the cell population as a whole.

Cold Shocking Method

The synchronizing effect of chilling has been established for many aerobic sporeformers as well as nonsporulating aerobic species. Much of the information obtained to date supports the hypothesis that chilling and rewarming affect DNA, RNA, and protein syntheses in a different manner. Falcone and Szybalski (1956) found that 30 to 60 minutes of chilling actively multiplying cells of B. megaterium at 15 C induced synchronized divisions during subsequent incubation at 34 C. Cell division was completely arrested after 30 minutes of cold shocking; DNA synthesis was least affected; RNA and protein synthesis were influenced the most by the decrease in temperature. The highest degree of induced

synchronization corresponded to (i) the period of largest difference between DNA and protein synthesis and (ii) the time immediately following the complete arrest of cell multiplcation. The authors postulated that the synchronization process might therefore depend on a limited period of unbalanced growth which, by favoring DNA synthesis, permitted the chromosomes in the non-dividing cells to terminate replication at the same locus. Under these circumstances, a new round of replication could not be initiated until the cells were restored to the warmer temperature and RNA and protein synthesis resumed. It is obvious that for this model of events the time of chilling is important. Supporting evidence is given by the studies of McNair Scott and Chu using E. coli as the test organism. These investigators found that 30 to 60 minutes at 4.5-6 C accomplished synchronized divisions during subsequent incubation at 36-37 C.

The present investigation indicates that the synchronization mechanism(s) of the cold shocking method applies also to Clostridium botulinum, type F, strain Langeland, an anaerobic sporeformer. A comparison of Figure 9 and 10 shows that (i) synchronized divisions can be induced in actively multiplying cells of the type F organism by 45 minutes of chilling at 4 C followed by incubation at 30 C, and (ii) cells of the type F organism are differentially susceptible to cold shocking; a short period of chilling applied to actively growing cultures (exponential phase) results in synchronized divisions whereas cold shocking stationary phase populations indicates only a tendency towards synchrony.

CHAPTER V

CONCLUSIONS

The investigation shows the following results:

1. Synchronous growth of vegetative cells of Clostridium botulinum, type F, strain Langeland may be obtained by germination synchrony of a spore inoculum in TSB^{+} at 30 C.

2. No synchronous growth is attained using the stationary phase method. An explanation, other than lack of technique, might be sought by employing the method of germination synchrony to investigate the synthesis, transcription, and replication of DNA in Clostridium botulinum, type F, strain Langeland.

3. Synchronized growth may be achieved by cold shocking exponentially growing, asynchronous cultures for 45 minutes at 4 C and returning them to fresh TSB^{+} at 30 C.

4. From the standpoints of reproducibility and time-saving, it proved essential to incorporate into the experimental procedure a Coulter Counter for total particle counts and a Technicon Auto-Analyzer for the continuous measurement of optical densities.

In the first method, germination may be sufficiently well synchronized to aid in the study of the initiation of DNA transcription and translation at the time when dormant spores of Clostridium botulinum, type F, strain Langeland make the transition to vegetative cells. Furthermore, the degree of cellular synchrony following outgrowth of the spores may be sufficient to permit the delineation of events which lead to the

formation of the toxin molecule. The method and medium, however, seem to be ill suited for studies on the initiation and regulation of spore formation since sporulation does not ensue until 3 to 4 days after cultures reach the stationary phase of growth.

The synchronized growth, as a result of cold shocking, is not expected to yield information on how spores and toxin molecules of Clostridium botulinum type F, strain Langeland are formed under normal cultural conditions. But, ancillary to a selective synchronization method, the study of synchronized growth may show the effect of a physical agent on the sequence of events which lead to spore and toxin formation. Furthermore, synchronized growth may be utilized in studies on the interrelationships of DNA and cell division, and RNA and protein synthesis as described in the introduction.

CHAPTER VI

RECOMMENDATIONS

Based on the results of this investigation, the following is recommended for further consideration:

1. A more detailed (or intensive) study of the factors which induce rapid germination of spores of Clostridium botulinum, type F, strain Langeland such as heat shocking (temperature, length of time) and composition of germination medium. These factors have been elucidated for some species of aerobic sporeformers. As a result, the germination of these organisms within 3 to 10 minutes is followed by several distinct synchronous generations of vegetative cells.

2. A study of the factors which induce and enhance sporulation. This involves compounding a suitable sporulation medium, a project which might easily be a major research effort in itself. But success could lead to studies on the induction and regulation of spore formation in Clostridium botulinum, type F by methods which have been described earlier in this text as applicable to Clostridium botulinum, type A and Clostridium roseum.

3. Utilization of the present method of germination synchrony as a basis for possible research support from public health agencies with the objective of delineating the events of toxin formation.

4. Extension of the use of the Technicon Auto-Analyzer to automatic and continuous determinations of DNA, RNA, and total protein throughout the growth cycle of Clostridium botulinum as well as those of other organisms.

APPENDICES

APPENDIX A

Spore Stain

Small samples were taken from a culture at varying times during its growth cycle and spread on clean slides. The smears were air dried and fixed with heat by passing the slides through a flame two to three times. They were then flooded with a 5 percent (w/v) aqueous solution of malachite green and heated until the stain steamed for 5 minutes. The staining solution was prevented from drying during that time by periodic addition of more stain as needed. A water rinse followed, and safranin (safranin O: a 0.5 percent solution in 95 percent ethyl alcohol, 10 ml; distilled water, 100 ml) was applied as a counterstain for one minute.

Vegetative cells appeared red in color and the spores as green bodies in a terminal to subterminal position, oval in shape, and swelling the vegetative cells into the club-shaped form that is characteristic of Clostridium botulinum.

Germinating spores, having lost their affinity for malachite green, were stained by flooding a heat fixed smear with methylene blue (methylene blue, 0.3g; 95 percent ethyl alcohol, 30 ml; distilled water, 100 ml) for two minutes.

Gram Stain

A drop of a culture from the early exponential phase of growth was placed on a clean slide and allowed to air dry (the Gram reaction of Clostridium botulinum may change to Gram variable or even Gram negative

as a culture becomes progressively older). The smear was fixed with heat and then flooded with Hucker's ammonium oxalate crystal violet.*

After 1-2 minutes of staining the slide was rinsed with water and flooded with Gram's iodine (iodine, 1.0g; KI, 2.0g; distilled water, 300 ml) for one minute followed by another two minutes with fresh Gram's iodine. The slide was then rinsed with water, flooded with 95 percent ethyl alcohol for 20 seconds and immediately rinsed with water again. Safranin O was now applied as a counterstain. The stained preparation was dried and examined microscopically.

Type C Toxin Medium (Cardella et al., 1958)

Proteose Peptone (Difco)	40 g
N-Z-Amine Type B (Sheffield Farms)	20 "
Yeast Extract (BBL)	20 "
Dextrose	10 "
Deionized water	950 ml

The ingredients (except dextrose) were added to the deionized water and the mixture heated with constant stirring until the nutrients were dissolved. After cooling to room temperature, the pH was adjusted to 7.2 with 10 N NaOH and the medium dispensed into Erlenmeyer flasks. These were plugged with cotton wrapped in one layer of cheese-cloth. The plugs were covered with aluminum foil to prevent them from being blown off the flasks during autoclaving. The medium was autoclaved at 120 C and 15 psig for 30 minutes and then rapidly cooled in an ice bath until warm to the

*Manual of Microbiological Methods, 1957, p. 16, McGraw-Hill Book Co., Inc. New York.

touch. Filter-sterilized dextrose was added aseptically just prior to inoculation to give a final concentration of one percent and the flasks gently swirled in order to distr b te the dextrose throughout the medium.

Spore Cleaning Procedure

Stock Solutions

Trypsin 1:250 (Difco)..... 5 mg/ml

Lysozyme (6000-10,000 units/mg)*..... 10 mg/ml

These were filter-sterilized and kept under refrigeration. The following mixture was dispensed aseptically into a sterile 150 ml beaker:

Spore stock..... 2.0 ml

Trypsin solution..... 1.0 "

Lysozyme solution..... 1.0 "

Deionized water..... 46.0 "

The solution was then subjected to an alternating schedule of sonic treatment (Biosonik III** intensity setting of 50) and digestion with trypsin and lysozyme at 45 C:

1. Sonication---------- 5 min.
2. Digestion---------- 30 "
3. Sonication---------- 5 "
4. Digestion---------- 30 "
5. Sonication---------- 5 "
6. Digestion---------- 60 "
7. Sonication---------- 5 "

During sonication the beaker was placed in a sterile Myler bag and the

*Nutritional Biochemical Corp., Cleveland, Ohio.
**Bronwill Scientific, Rochester, New York.

sonic probe inserted through an opening in the plastic to prevent aerosol from escaping.

After completion of the treatment, the spores were spun at 2000 x g for 30 minutes, followed by three washings with cold, sterile, deionized water. Finally, the cleaned spores were resuspended in as small an amount of sterile deionized water as possible and stored at 4 C.

Peptone Water

Bacto-peptone..... 1 g

Deionized water..... 1000 ml

The peptone was dissolved in water and 99 ml aliquots dispensed into 150 ml milk dilution bottles. After autoclaving for 20 minutes at 120 C and 15 psig, the bottles were stoppered aseptically with sterile rubber stoppers and stored at room temperature.

Fresh Pork Infusion Medium

First Day

A fresh pork ham (10-12 lbs.) was trimmed of as much fat as possible and ground in a meat grinder with a fine grinding attachment. The meat was mixed with distilled water in a ratio of one pound of pork per liter and the mixture brought to a boil with occasional stirring.

Simmering for two hours was followed by filtration through several layers of cheese-cloth to remove the meat particles. The pork infusion broth was cooled overnight in a refrigerator at 4 C to allow the fat to solidify.

Second Day

The fat was carefully skimmed off and the medium brought to its

original volume. The following additions were then made:

Bacto-peptone (Difco).....	5.0 g/l
Bacto-tryptone (Difco).....	1.5 "
K_2HPO_4.....	1.3 "
Dextrose.....	1.0 "
Soluble starch.....	1.0 "
Sodium thioglycollate.....	1.0 "

The ingredients were dissolved by heating the broth while stirring frequently. After cooling to room temperature, the pH was adjusted to 7.4 by adding 10 N NaOH.

The medium was now dispensed into 4 liter Erlenmeyer flasks and 1.5 percent (w/v) Bacto-Agar added to each flask. After cotton plugging the latter and covering the plugs with aluminum foil, the medium was autoclaved at 120 C and 15 psig for 30 minutes. The flasks were then placed in a 55 C water-bath in a tilted position and left overnight to allow the settling of precipitates.

Third Day

The cleared pork medium was carefully poured off and filtered through a 1/2 inch thick layer of cotton sandwiched between four layers of cheese-cloth. Prickett tubes (Fisher modified agar slant tubes) were filled with approximately 25 ml of the medium, cotton plugged, and autoclaved at 120 C and 15 psig for 30 minutes. It was necessary to cover the tops of the tubes with a thin piece of plywood weighted down with a brick in order to prevent the cotton plugs from blowing off during autoclaving.

APPENDIX B

Further efforts to synchronize the vegetative growth of Clostridium botulinum, type F, strain Langeland by the stationary phase method consisted of: (i) changing the procedure described by Cutler and Evans (1966) by omitting the streak plates and instead following the procedure described earlier in this text for cold shocking stationary phase cultures, and (ii) using a synthetic rather than undefined medium. It was noted that the successful synchronizations of vegetative growth by the stationary phase method were accomplished in defined minimum glucose-salts media. Assuming that such a minimum medium might favor the synchronization mechanism(s), I attempted to grow Clostridium botulinum, type F in a synthetic medium. The only literature reference available on the type F organism was that of Haldeman Holdeman (1964) who was successful in growing certain laboratory variants of the Langeland strain in a synthetic medium. The ingredients with the amounts of each she found essential for the growth of the laboratory variants are shown in Table 7. The Langeland strain adjusted to the medium after several weeks of daily transfers but growth, as measured by turbidity, was not sufficient for experimentation.

Table 7. Composition of Synthetic Medium for the Growth of Clostridium botulinum, Type F, Strain Langeland.

Ingredient	Amount/100 ml
K_2HPO_4	50.00 mg
KH_2PO_4	50.00 mg
NaCl	1.00 mg
$MgSO_4$	20.00 mg
$FeSO_4 \cdot H_2O$	1.00 mg
$MnSO_4 \cdot H_2O$	0.82 mg
sodium thioglycollate	100.00 mg
glucose	500.00 mg
DL isolencine	50.00 mg
DL phenylalanine	200.00 mg
DL tryptophan	5.00 mg
DL valine	200.00 mg
L arginine	300.00 mg
L tyrosine	25.00 mg
glycine	10.00 mg
biotin	0.05 mcg
para-aminobenzoic acid	1.00 mcg
thiamin HCl	50.00 mcg

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